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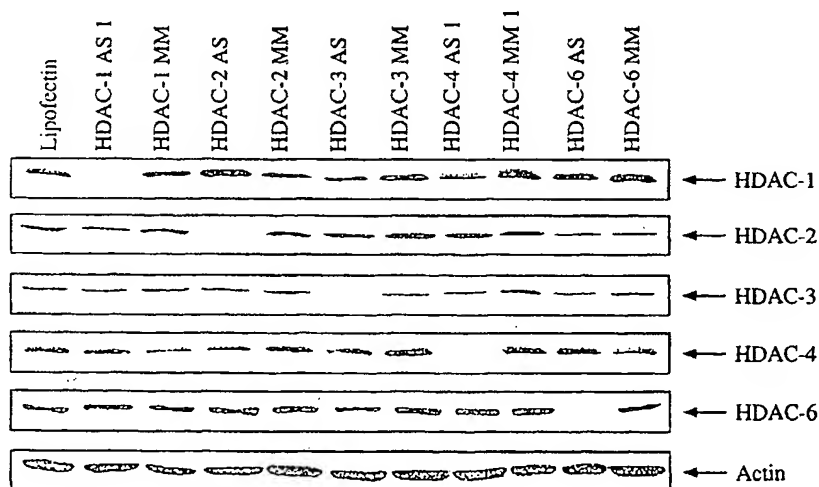
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(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS



AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc - 50nM

(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

5 BACKGROUND OF THE INVENTION

This invention relates to the fields of inhibition of histone deacetylase expression and enzymatic activity.

In eukaryotic cells, nuclear DNA associates with histones to form a compact complex called chromatin. The histones constitute a family of basic proteins which are generally highly conserved across eukaryotic species. The core histones, termed H2A, H2B, H3, and H4, associate to form a protein core. DNA winds around this protein core, with the basic amino acids of the histones interacting with the negatively charged phosphate groups of the DNA. Approximately 146 base pairs of DNA wrap around a histone core to make up a nucleosome particle, the repeating structural motif of chromatin.

Csordas, *Biochem. J.*, 286: 23-38 (1990) teaches that histones are subject to posttranslational acetylation of the epsilon-amino groups of N-terminal lysine residues, a reaction that is catalyzed by histone acetyltransferase (HAT1). Acetylation neutralizes the positive charge of the lysine side chain, and is thought to impact chromatin structure. Indeed, Taunton *et al.*, *Science*, 272: 408-411 (1996), teaches that access of transcription factors to chromatin templates is enhanced by histone hyperacetylation. Taunton *et al.* further teaches that an enrichment in underacetylated histone H4 has been found in transcriptionally silent regions of the genome.

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Recently, there has been interest in the role of histone deacetylase (HDAC) in gene expression. Sanches Del Pino *et al.*, *Biochem. J.* 303: 723-729 (1994) discloses a partially purified yeast HDAC activity. Taunton *et al.* (*supra*) discloses a human HDAC that is related to a yeast transcriptional
5 regulator and suggests that this protein may be a key regulator of eukaryotic transcription.

Known inhibitors of mammalian HDAC have been used to probe the role of HDAC in gene regulation. Yoshida *et al.*, *J. Biol. Chem* 265: 17174-17179 (1990) discloses that (R)-Trichostatin A (TSA) is a potent
10 inhibitor of mammalian HDAC. Yoshida *et al.*, *Cancer Research* 47: 3688-3691 (1987) discloses that TSA is a potent inducer of differentiation in murine erythroleukemia cells.

More recently, it has been discovered that the HDAC activity is actually provided by a set of discrete HDAC enzyme isoforms. Grozinger
15 *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 96: 4868-4873 (1999), teaches that HDACs may be divided into two classes, the first represented by yeast Rpd3-like proteins, and the second represented by yeast Hda1-like proteins. Grozinger *et al.* also teaches that the human HDAC1, HDAC2, and HDAC3 proteins are members of the first class of HDACs, and discloses new
20 proteins, named HDAC4, HDAC5, and HDAC6, which are members of the second class of HDACs. Kao *et al.*, *Gene & Development* 14: 55-66 (2000), discloses an additional member of this second class, called HDAC-7. More recently, Hu, E. *et al.*, *J. Bio. Chem.* 275:15254-13264 (2000) disclosed the newest member of the first class of histone deacetylases, HDAC-8. It has
25 been unclear what roles these individual HDAC enzymes play.

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The known inhibitors of histone deacetylase are all small molecules that inhibit histone deacetylase activity at the protein level. Moreover, all of the known histone deacetylase inhibitors are non-specific for a particular histone deacetylase isoform, and more or less inhibit all members of both

5 the histone deacetylase families equally.

Therefore, there remains a need to develop reagents for inhibiting specific histone deacetylase isoforms. There is also a need for the development of methods for using these reagents to identify and inhibit specific histone deacetylase isoforms involved in tumorigenesis.

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BRIEF SUMMARY OF THE INVENTION

The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The inventors have discovered new agents that inhibit specific HDAC isoforms. Accordingly, in a first aspect, the invention provides agents that inhibit one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms. Such specific HDAC isoforms
15 include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. Non-limiting examples of the new agents include antisense oligonucleotides (oligos) and small molecule inhibitors specific for one or more HDAC isoforms but less than all HDAC isoforms.

20 The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the
25 invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

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In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding that histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (e.g., a gene), cDNA, or RNA. In some
5 embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC isoform. In other embodiments, the oligonucleotide inhibits translation of the histone deacetylase isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Particularly preferred embodiments include antisense
10 oligonucleotides directed to HDAC-1 and/or HDAC-4.

In yet other embodiments of the first aspect, the agent that inhibits a specific HDAC isoform is a small molecule inhibitor that inhibits the activity of one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms.

15 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell, comprising contacting the cell with an agent of the first aspect of the invention. In other preferred embodiments, the agent is an antisense oligonucleotide. In certain preferred embodiments, the agent is a small
20 molecule inhibitor. In other certain preferred embodiments of the second aspect of the invention, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method of the second aspect of the
25 invention further comprises contacting the cell with a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of one or more specific histone deacetylase isoforms. In still yet other preferred embodiments of the second aspect of the invention, the method comprises an agent of the first aspect of the invention which is a

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combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In
5 other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4. In some embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide.

In a third aspect, the invention provides a method for inhibiting
10 neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide which is combined with a pharmaceutically acceptable
15 carrier and administered for a therapeutically effective period of time. In certain preferred embodiments, the agent is a small molecule inhibitor which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain preferred embodiments of the this aspect of the invention, cell proliferation
20 is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In other certain embodiments, the agent is a small molecule inhibitor of the first aspect of the invention which is combined with a pharmaceutically acceptable carrier and administered for
25 a therapeutically effective period of time. In still yet other preferred embodiments of the third aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred

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embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

- 5 In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone
- 10 deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that
- 15 inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is
- 20 a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In
- 25 certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of

5 differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is an small molecule inhibitor of

10 the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first

15 aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting

20 neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for

25 a therapeutically effective period of time.

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In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide from the first aspect of the invention that inhibits expression of a specific histone deacetylase isoform, a small molecule inhibitor from the first aspect of the invention that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a small molecule that inhibits a DNA methyltransferase. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation, comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In certain embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In preferred embodiments, the cell proliferation is neoplasia. In still yet other preferred embodiments of the this aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic diagram providing the amino acid sequence of HDAC-1, as provided in GenBank Accession No. AAC50475 (SEQ ID NO:1).

5

Figure 1B is a schematic diagram providing the nucleic acid sequence of HDAC-1, as provided in GenBank Accession No. U50079 (SEQ ID NO:2).

10

Figure 2A is a schematic diagram providing the amino acid sequence of HDAC-2, as provided in GenBank Accession No. AAC50814 (SEQ ID NO:3).

15

Figure 2B is a schematic diagram providing the nucleic acid sequence of HDAC-2, as provided in GenBank Accession No. U31814 (SEQ ID NO:4).

20

Figure 3A is a schematic diagram providing the amino acid sequence of HDAC-3, as provided in GenBank Accession No. AAB88241 (SEQ ID NO:5).

25

Figure 3B is a schematic diagram providing the nucleic acid sequence of HDAC-3, as provided in GenBank Accession No. U75697 (SEQ ID NO:6).

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Figure 4A is a schematic diagram providing the amino acid sequence of HDAC-4, as provided in GenBank Accession No. BAA22957 (SEQ ID NO:7).

5 Figure 4B is a schematic diagram providing the nucleic acid sequence of HDAC-4, as provided in GenBank Accession No. AB006626 (SEQ ID NO:8).

10 Figure 5A is a schematic diagram providing the amino acid sequence of HDAC-5, as provided in GenBank Accession No. BAA25526 (SEQ ID NO:9).

15 Figure 5B is a schematic diagram providing the nucleic acid sequence of HDAC-5 as provided in GenBank Accession No. AB011172 (SEQ ID NO:10).

20 Figure 6A is a schematic diagram providing the amino acid sequence of human HDAC-6, as provided in GenBank Accession No. AAD29048 (SEQ ID NO:11).

Figure 6B is a schematic diagram providing the nucleic acid sequence of human HDAC-6, as provided in GenBank Accession No. AJ011972 (SEQ ID NO:12).

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Figure 7A is a schematic diagram providing the amino acid sequence of human HDAC-7, as provided in GenBank Accession No. AAF63491.1 (SEQ ID NO:13).

5 Figure 7B is a schematic diagram providing the nucleic acid sequence of human HDAC-7, as provided in GenBank Accession No. AF239243 (SEQ ID NO:14).

10 Figure 8A is a schematic diagram providing the amino acid sequence of human HDAC-8, as provided in GenBank Accession No. AAF73076.1 (SEQ ID NO:15).

15 Figure 8B is a schematic diagram providing the nucleic acid sequence of human HDAC-8, as provided in GenBank Accession No. AF230097 (SEQ ID NO:16).

20 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-1 AS1 antisense oligonucleotide on HDAC-1 mRNA expression in human A549 cells.

25 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-2 AS antisense oligonucleotide on HDAC-2 mRNA expression in human A549 cells.

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Figure 9C is a representation of a Northern blot demonstrating the effect of HDAC-6 AS antisense oligonucleotide on HDAC-6 mRNA expression in human A549 cells.

5 Figure 9D is a representation of a Northern blot demonstrating the effect of HDAC-3 AS antisense oligonucleotide on HDAC-3 mRNA expression in human A549 cells.

10 Figure 9E is a representation of a Northern blot demonstrating the effect of an HDAC-4 antisense oligonucleotide (AS1) on HDAC-4 mRNA expression in human A549 cells.

15 Figure 9F is a representation of a Northern blot demonstrating the dose-dependent effect of an HDAC-4 antisense oligonucleotide (AS2) on HDAC-4 mRNA expression in human A549 cells.

20 Figure 9G is a representation of a Northern blot demonstrating the effect of an HDAC-5 antisense oligonucleotide (AS) on HDAC-5 mRNA expression in human A549 cells.

 Figure 9H is a representation of a Northern blot demonstrating the effect of an HDAC-7 antisense oligonucleotide (AS) on HDAC-7 mRNA expression in human A549 cells.

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Figure 9I is a representation of a Northern blot demonstrating the dose-dependent effect of HDAC-8 antisense oligonucleotides (AS1 and AS2) on HDAC-8 mRNA expression in human A549 cells.

5 Figure 10A is a representation of a Western blot demonstrating the effect of HDAC isotype-specific antisense oligos on HDAC isotype protein expression in human A549 cells.

10 Figure 10B is a representation of a Western blot demonstrating the dose-dependent effect of the HDAC-1 isotype-specific antisense oligo (AS1 and AS2) on HDAC isotype protein expression in human A549 cells.

15 Figure 10C is a representation of a Western blot demonstrating the effect of HDAC-4 isotype-specific antisense oligonucleotide (AS2) on HDAC isotype protein expression in human A549 cells.

20 Figure 11A is a graphic representation demonstrating the apoptotic effect of HDAC isotype-specific antisense oligos on human A549 cancer cells.

Figure 12A is a graphic representation demonstrating the effect of HDAC-1 AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

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Figure 12B is a graphic representation demonstrating the effect of HDAC-8 specific AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

5 Figure 13 is a a graphic representation demonstrating the cell cycle blocking effect of HDAC specific antisense oligonucleotides on human A549 cancer cells.

10 Figure 14 is a representation of an RNase protection assay demonstrating the effect of HDAC isotype-specific antisense oligonucleotides on HDAC isotype mRNA expression in human A549 cells.

15 Figure 15 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-4 AS1 antisense oligonucleotide induces the expression of the p21 protein.

20 Figure 16 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-1 antisense oligonucleotides (AS1 and AS2) represses the expression of the cyclin B1 and cyclin A genes.

25 Figure 17 shows plating data demonstrating the ability of antisense oligonucleotides complementary to HDAC-1 to inhibit growth in soft agar of A549 cells far more than can antisense oligonucleotides complementary to HDAC-2, HDAC-6 or mismatched controls.

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Figure 18 is a representation of a Western blot demonstrating that treatment of human A549 cells with the small molecule inhibitor Compound 3 (Table 2) induces the expression of the p21 protein and represses the expression of the cyclin B1 and cyclin A genes.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides methods and reagents for inhibiting specific histone deacetylase isoforms (HDAC) by inhibiting expression at the nucleic acid level or protein activity at the enzymatic level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the
15 same extent as if each was specifically and individually indicated to be incorporated by reference.

In a first aspect, the invention provides agents that inhibit one or more histone deacetylase isoform, but less than all specific histone deacetylase isoforms. As used herein interchangeably, the terms "histone deacetylase", "HDAC", "histone deacetylase isoform", "HDAC isoform" and
20 similar terms are intended to refer to any one of a family of enzymes that remove acetyl groups from the epsilon-amino groups of lysine residues at the *N*-terminus of a histone. Unless otherwise indicated by context, the term "histone" is meant to refer to any histone protein, including H1, H2A, H2B, H3, and H4, from any species. Preferred histone deacetylase isoforms
25 include class I and class II enzymes. Specific HDACs include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. By way of non-limiting example, useful agents that

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inhibit one or more histone deacetylase isoforms, but less than all specific histone deacetylase isoforms, include antisense oligonucleotides and small molecule inhibitors.

The present inventors have surprisingly discovered that specific
5 inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the invention, the histone deacetylase isoform that is inhibited is HDAC-1
10 and/or HDAC-4.

Preferred agents that inhibit HDAC-1 and/or HDAC-4 dramatically inhibit growth of human cancer cells, independent of p53 status. These agents significantly induce apoptosis in the cancer cells and cause dramatic growth arrest. They also can induce transcription of tumor suppressor
15 genes, such as p21^{WAF1}, p57^{KIP2}, GADD153 and GADD45. Finally, they exhibit both *in vitro* and *in vivo* anti-tumor activity. Inhibitory agents that achieve one or more of these results are considered within the scope of this aspect of the invention. By way of non-limiting example, antisense oligonucleotides and/or small molecule inhibitors of HDAC-1 and/or
20 HDAC-4 are useful for the invention.

In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding a specific histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA. In
25 other embodiments, the oligonucleotide ultimately inhibits translation of the histone deacetylase. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Preferred antisense

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oligonucleotides have potent and specific antisense activity at nanomolar concentrations.

The antisense oligonucleotides according to the invention are complementary to a region of RNA or double-stranded DNA that encodes
5 a portion of one or more histone deacetylase isoform (taking into account that homology between different isoforms may allow a single antisense oligonucleotide to be complementary to a portion of more than one isoform).

For purposes of the invention, the term "complementary" means
10 having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base
15 stacking can lead to hybridization. As a practical matter, such hybridization can be inferred from the observation of specific gene expression inhibition, which may be at the level of transcription or translation (or both).

For purposes of the invention, the term "oligonucleotide" includes
20 polymers of two or more deoxyribonucleosides, ribonucleosides, or 2'-O-substituted ribonucleoside residues, or any combination thereof. Preferably, such oligonucleotides have from about 8 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The nucleoside residues may be coupled to each other by any of
25 the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate,

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carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleotide linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or
5 phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines, and adamantane. The term oligonucleotide also encompasses such polymers as
10 PNA and LNA. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with an -O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl, or allyl group may be unsubstituted or may be substituted, *e.g.*, with
15 halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group.

Particularly preferred antisense oligonucleotides utilized in this
20 aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

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For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or
5 phosphorodithioate region, preferably comprising from about 2 to about 12 nucleotides, and an alkylphosphonate or alkylphosphonothioate region (see *e.g.*, Pederson *et al.* U.S. Patent Nos. 5,635,377 and 5,366,878). Preferably, such chimeric oligonucleotides contain at least three consecutive internucleoside linkages selected from phosphodiester and
10 phosphorothioate linkages, or combinations thereof.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred embodiment of such a hybrid oligonucleotide comprises a
ribonucleotide or 2'-O-substituted ribonucleotide region, preferably
15 comprising from about 2 to about 12 2'-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide will contain at least three consecutive deoxyribonucleosides and will also contain ribonucleosides, 2'-O-substituted ribonucleosides, or combinations thereof (see *e.g.*, Metelev and Agrawal, U.S. Patents Nos. 5,652,355 and
20 5,652,356).

The exact nucleotide sequence and chemical structure of an antisense oligonucleotide utilized in the invention can be varied, so long as the oligonucleotide retains its ability to inhibit expression of a specific histone deacetylase isoform or inhibit one or more histone deacetylase
25 isoforms, but less than all specific histone deacetylase isoforms. This is readily determined by testing whether the particular antisense oligonucleotide is active by quantitating the amount of mRNA encoding a specific histone deacetylase isoform, quantitating the amount of histone

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deacetylase isoform protein, quantitating the histone deacetylase isoform enzymatic activity, or quantitating the ability of the histone deacetylase isoform to inhibit cell growth in a an *in vitro* or *in vivo* cell growth assay, all of which are described in detail in this specification. The term "inhibit
5 expression" and similar terms used herein are intended to encompass any one or more of these parameters.

Antisense oligonucleotides utilized in the invention may conveniently be synthesized on a suitable solid support using well-known chemical approaches, including H-phosphonate chemistry,
10 phosphoramidite chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase oligonucleotide synthesis, such as controlled-pore glass (CPG) (see, *e.g.*,
15 Pon, R. T., Methods in Molec. Biol. 20: 465-496, 1993).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of specific histone deacetylase isoforms by being used to inhibit the activity of specific histone deacetylase isoforms in an
20 experimental cell culture or animal system and to evaluate the effect of inhibiting such specific histone deacetylase isoform activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide that inhibits one or more histone deacetylase isoform expression according to the invention and observing any phenotypic
25 effects. In this use, the antisense oligonucleotides according to the invention is preferable to traditional "gene knockout" approaches because it is easier to use, and can be used to inhibit specific histone deacetylase isoform activity at selected stages of development or differentiation.

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- Preferred antisense oligonucleotides of the invention inhibit either the transcription of a nucleic acid molecule encoding the histone deacetylase isoform, and/or the translation of a nucleic acid molecule encoding the histone deacetylase isoform, and/or lead to the degradation of such nucleic acid. Histone deacetylase-encoding nucleic acids may be RNA or double stranded DNA regions and include, without limitation, intronic sequences, untranslated 5' and 3' regions, intron-exon boundaries as well as coding sequences from a histone deacetylase family member gene. For human sequences, see *e.g.*, Yang et al., *Proc. Natl. Acad. Sci. (USA)* 93(23): 12845-12850, 1996; Furukawa et al., *Cytogenet. Cell Genet.* 73(1-2): 130-133, 1996; Yang et al., *J. Biol. Chem.* 272(44): 28001-28007, 1997; Betz et al., *Genomics* 52(2): 245-246, 1998; Taunton et al., *Science* 272(5260): 408-411, 1996; and Dangond et al., *Biochem. Biophys. Res. Commun.* 242(3): 648-652, 1998).
- Particularly preferred non-limiting examples of antisense oligonucleotides of the invention are complementary to regions of RNA or double-stranded DNA encoding a histone deacetylase isoform (*e.g.*, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8). (see *e.g.*, GenBank Accession No. U50079 for human HDAC-1 (Fig. 1B); GenBank Accession No. U31814 for human HDAC-2; (Fig. 2B) GenBank Accession No. U75697 for human HDAC-3 (Fig. 3B; GenBank Accession No. AB006626 for human HDAC-4 (Fig. 4B); GenBank Accession No. AB011172 for human HDAC-5 (Fig. 5B); GenBank Accession No. AJ011972 for human HDAC-6 (Fig. 6B); GenBank Accession No. AF239243 for human HDAC-7 (Fig. 7B); and GenBank Accession No. AF230097 for human HDAC-8 (Fig. 8B)).

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The sequences encoding histone deacetylases from many non-human animal species are also known (see, for example, GenBank Accession Numbers X98207 (murine HDAC-1); NM_008229 (murine HDAC-2); NM_010411 (murine HDAC-3); NM_006037 (murine HDAC-4);
5 NM_010412 (murine HDAC-5); NM_010413 (murine HDAC-6); and AF207749 (murine HDAC-7)). Accordingly, the antisense oligonucleotides of the invention may also be complementary to regions of RNA or double-stranded DNA that encode histone deacetylases from non-human animals. Antisense oligonucleotides according to these embodiments are useful as
10 tools in animal models for studying the role of specific histone deacetylase isoforms.

Particularly, preferred oligonucleotides have nucleotide sequences of from about 13 to about 35 nucleotides which include the nucleotide sequences shown in Table I. Yet additional particularly preferred
15 oligonucleotides have nucleotide sequences of from about 15 to about 26 nucleotides of the nucleotide sequences shown below. Most preferably, the oligonucleotides shown below have phosphorothioate backbones, are 20-26 nucleotides in length, and are modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four
20 nucleotides at the 3' end of the oligonucleotide each have 2'-O- methyl groups attached to their sugar residues.

Antisense oligonucleotides used in the present study are shown in Table I.

Table 1

Sequences of Human Isotype-Specific Antisense (AS) Oligonucleotides and Their Mismatch (MM) Oligonucleotides

Oligo	Target	Accession Number	Nucleotide Position	Sequence	Gene Position
HDAC1 AS1	Human HDAC1	U50079	1585-1604	5'-GAAACGTGAGGGACTCAGCA-3' (SEQ ID NO:17)	3'-UTR
HDAC1 AS2	Human HDAC1	U50079	1565-1584	5'-GGAAGCCAGAGCTGGAGAGG-3' (SEQ ID NO:18)	3'-UTR
HDAC1 MM	Human HDAC1	U50079	1585-1604	5'-GTTAGGTGAGGCACTGAGGA-3' (SEQ ID NO:19)	3'-UTR
HDAC2 AS	Human HDAC2	U31814	1643-1622	5'-GCTGAGCTGTTCTGATTTGG-3' (SEQ ID NO:20)	3'-UTR
HDAC2 MM	Human HDAC2	U31814	1643-1622	5'-CGTGAGCACTTCTCATTTCC-3' (SEQ ID NO:21)	3'-UTR
HDAC3 AS	Human HDAC3	AF039703	1276-1295	5'-CGCTTTCCTTGTCATTGACA-3' (SEQ ID NO:22)	3'-UTR
HDAC3 MM	Human HDAC3	AF039703	1276-1295	5'-GCCTTTCCTACTCATTGTGT-3' (SEQ ID NO:23)	3'-UTR
HDAC4 AS1	Human HDAC4	AB006626	514-33	5'-GCTGCCTGCCGTGCCACCC-3' (SEQ ID NO:24)	5'-UTR
HDAC4 MM1	Human HDAC4	AB006626	514-33	5'-CGTGCCTGCGCTGCCACGG-3' (SEQ ID NO:25)	5'-UTR
HDAC4 AS2	Human HDAC4	AB006626	7710-29	5'-TACAGTCCATGCAACCTCCA-3' (SEQ ID NO:26)	3'-UTR
HDAC4 MM4	Human HDAC4	AB006626	7710-29	5'-ATCAGTCCAACCAACCTCGT-3' (SEQ ID NO:27)	3'-UTR
HDAC5 AS	Human HDAC5	AF039691	2663-2682	5'-CTTCGGTCTCACCTGCTTGG-3' (SEQ ID NO:28)	3'-UTR
HDAC6 AS	Human HDAC6	AJ011972	3791-3810	5'-CAGGCTGGAATGAGCTACAG-3' (SEQ ID NO:29)	3'-UTR
HDAC6 MM	Human HDAC6	AJ011972	3791-3810	5'-GACGCTGCAATCAGGTAGAC-3' (SEQ ID NO:30)	3'-UTR
HDAC7 AS	Human HDAC7	AF239243	2896-2915	5'-CTTCAGCCAGGATGCCACACA-3' (SEQ ID NO:31)	3'-UTR
HDAC8 AS1	Human HDAC8	AF230097	51-70	5'-CTCCGGCTCCTCCATCTTCC-3' (SEQ ID NO:32)	5'-UTR
HDAC8 AS2	Human HDAC8	AF230097	1328-1347	5'-AGCCAGCTGCCACTTGATGC-3' (SEQ ID NO:33)	3'-UTR

The antisense oligonucleotides according to the invention may optionally be formulated with any of the well known pharmaceutically acceptable carriers or diluents (see preparation of pharmaceutically acceptable formulations in, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990), with the proviso that such carriers or diluents not affect their ability to modulate HDAC activity.

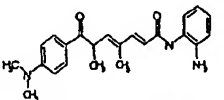
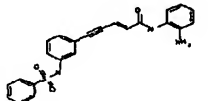
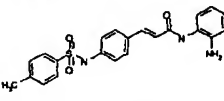
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By way of non-limiting example, the agent of the first aspect of the invention may also be a small molecule inhibitor. The term "small molecule" as used in reference to the inhibition of histone deacetylase is used to identify a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably less than 600 Da, which is capable of interacting with a histone deacetylase and inhibiting the expression of a nucleic acid molecule encoding an HDAC isoform or activity of an HDAC protein. Inhibiting histone deacetylase enzymatic activity means reducing the ability of a histone deacetylase to remove an acetyl group from a histone. In some preferred embodiments, such reduction of histone deacetylase activity is at least about 50%, more preferably at least about 75%, and still more preferably at least about 90%. In other preferred embodiments, histone deacetylase activity is reduced by at least 95% and more preferably by at least 99%. In one certain embodiment, the small molecule inhibitor is an inhibitor of one or more but less than all HDAC isoforms. By "all HDAC isoforms" is meant all proteins that specifically remove an epsilon acetyl group from an N-terminal lysine of a histone, and includes, without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8, all of which are considered "related proteins," as used herein.

Most preferably, a histone deacetylase small molecule inhibitor interacts with and reduces the activity of one or more histone deacetylase isoforms (*e.g.*, HDAC-1 and/or HDAC-4), but does not interact with or reduce the activities of all of the other histone deacetylase isoforms (*e.g.*, HDAC-2 and HDAC-6). As discussed below, a preferred histone deacetylase small molecule inhibitor is one that interacts with and reduces the enzymatic activity of a histone deacetylase isoform that is involved in tumorigenesis.

Non-limiting examples of small molecule inhibitors useful for the invention are presented in Table 2.

Table 2

Small Molecule HDAC Inhibitors [μ M] and Their Antitumor Activities <i>In Vivo</i>												
Cpd	Inhibitor Structure	Enzyme IC ₅₀ (μ M)						% inhibitor of tumor formation in vivo				
		HDAC1	HDAC2	HDAC3	HDAC4	HDAC6	H4-Ac	MTT	Cell Cycle Arrest EC	colon	lung	prostate
1		3	25	21	23	>50	1	3	2			
2		3	31	30	35	>30	5	4	8	53 (40,po)	54 (50,ip)	
3		3	22	45	28	>50	5	4	2	55 (40,ip)		
note: for <i>in vivo</i> antitumor studies, numbers outside brackets indicate % of inhibition of tumor growth in vivo; numbers in brackets indicate daily dose of inhibitor used (mg/kg body weight/day); oral (PO) or intraperitoneal (IP) administration is indicated in brackets.												

5 The reagents according to the invention are useful as analytical tools and as therapeutic tools, including as gene therapy tools. The invention also provides methods and compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects.

10 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell comprising contacting the cell with an agent of the first aspect of the invention. By way of non-limiting example, the agent may be an antisense oligonucleotide or a small molecule inhibitor that inhibits the expression of

15 one or more, but less than all, specific histone deacetylase isoforms in the cell.

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In one certain embodiment, the invention provides a method comprising contacting a cell with an antisense oligonucleotide that inhibits one or more but less than all histone deacetylase isoforms in the cell. Preferably, cell proliferation is inhibited in the contacted cell. Thus, the

5 antisense oligonucleotides according to the invention are useful in therapeutic approaches to human diseases including benign and malignant neoplasms by inhibiting cell proliferation in cells contacted with the antisense oligonucleotides. The phrase "inhibiting cell proliferation" is used to denote an ability of a histone deacetylase antisense oligonucleotide

10 or a small molecule histone deacetylase inhibitor (or combination thereof) to retard the growth of cells contacted with the oligonucleotide or small molecule inhibitor, as compared to cells not contacted. Such an assessment of cell proliferation can be made by counting contacted and non-contacted cells using a Coulter Cell Counter (Coulter, Miami, FL) or a

15 hemacytometer. Where the cells are in a solid growth (*e.g.*, a solid tumor or organ), such an assessment of cell proliferation can be made by measuring the growth with calipers, and comparing the size of the growth of contacted cells with non-contacted cells. Preferably, the term includes a retardation of cell proliferation that is at least 50% of non-contacted cells.

20 More preferably, the term includes a retardation of cell proliferation that is 100% of non-contacted cells (*i.e.*, the contacted cells do not increase in number or size). Most preferably, the term includes a reduction in the number or size of contacted cells, as compared to non-contacted cells. Thus, a histone deacetylase antisense oligonucleotide or a histone

25 deacetylase small molecule inhibitor that inhibits cell proliferation in a contacted cell may induce the contacted cell to undergo growth retardation, to undergo growth arrest, to undergo programmed cell death (*i.e.*, to apoptose), or to undergo necrotic cell death.

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Conversely, the phrase "inducing cell proliferation" and similar terms are used to denote the requirement of the presence or enzymatic activity of a specific histone deacetylase isoform for cell proliferation in a normal (*i.e.*, non-neoplastic) cell. Hence, over-expression of a specific histone deacetylase isoform that induces cell proliferation may or may not lead to increased cell proliferation; however, inhibition of a specific histone deacetylase isoform that induces cell proliferation will lead to inhibition of cell proliferation.

The cell proliferation inhibiting ability of the antisense oligonucleotides according to the invention allows the synchronization of a population of a-synchronously growing cells. For example, the antisense oligonucleotides of the invention may be used to arrest a population of non-neoplastic cells grown *in vitro* in the G1 or G2 phase of the cell cycle. Such synchronization allows, for example, the identification of gene and/or gene products expressed during the G1 or G2 phase of the cell cycle. Such a synchronization of cultured cells may also be useful for testing the efficacy of a new transfection protocol, where transfection efficiency varies and is dependent upon the particular cell cycle phase of the cell to be transfected. Use of the antisense oligonucleotides of the invention allows the synchronization of a population of cells, thereby aiding detection of enhanced transfection efficiency.

The anti-neoplastic utility of the antisense oligonucleotides according to the invention is described in detail elsewhere in this specification.

In yet other preferred embodiments, the cell contacted with a histone deacetylase antisense oligonucleotide is also contacted with a histone deacetylase small molecule inhibitor.

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- In a few preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide. As mentioned above, the antisense oligonucleotides according to the invention may optionally be formulated well known pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more one or more additional histone deacetylase antisense oligonucleotide(s), and/or one or more histone deacetylase small molecule inhibitor(s), or it may contain any other pharmacologically active agent.
- 10 In a particularly preferred embodiment of the invention, the antisense oligonucleotide is in operable association with a histone deacetylase small molecule inhibitor. The term "operable association" includes any association between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor which allows an antisense
- 15 oligonucleotide to inhibit one or more specific histone deacetylase isoform-encoding nucleic acid expression and allows the histone deacetylase small molecule inhibitor to inhibit specific histone deacetylase isoform enzymatic activity. One or more antisense oligonucleotide of the invention may be operably associated with one or more histone deacetylase small molecule
- 20 inhibitor. In some preferred embodiments, an antisense oligonucleotide of the invention that targets one particular histone deacetylase isoform (*e.g.*, HDAC-1) is operably associated with a histone deacetylase small molecule inhibitor which targets the same histone deacetylase isoform. A preferred operable association is a hydrolyzable. Preferably, the hydrolyzable
- 25 association is a covalent linkage between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor. Preferably, such covalent linkage is hydrolyzable by esterases and/or amidases. Examples of such hydrolyzable associations are well known in the art. Phosphate esters are particularly preferred.

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In certain preferred embodiments, the covalent linkage may be directly between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor so as to integrate the histone deacetylase small molecule inhibitor into the backbone. Alternatively, the covalent linkage
5 may be through an extended structure and may be formed by covalently linking the antisense oligonucleotide to the histone deacetylase small molecule inhibitor through coupling of both the antisense oligonucleotide and the histone deacetylase small molecule inhibitor to a carrier molecule such as a carbohydrate, a peptide or a lipid or a glycolipid. Other
10 preferred operable associations include lipophilic association, such as formation of a liposome containing an antisense oligonucleotide and the histone deacetylase small molecule inhibitor covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphatidylcholine, cholesterol,
15 phosphatidylethanolamine, and synthetic neoglycolipids, such as squalylacNAc-HDPE. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when the antisense oligonucleotide is associated with one liposome and the small molecule inhibitor is associated
20 with another liposome.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the
25 invention. In one certain embodiment, the agent is an antisense oligonucleotide of the first aspect of the invention, and the method further comprises a pharmaceutically acceptable carrier. The antisense oligonucleotide and the pharmaceutically acceptable carrier are administered for a therapeutically effective period of time. Preferably, the

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animal is a mammal, particularly a domesticated mammal. Most preferably, the animal is a human.

The term "neoplastic cell" is used to denote a cell that shows aberrant cell growth. Preferably, the aberrant cell growth of a neoplastic cell is increased cell growth. A neoplastic cell may be a hyperplastic cell, a
5 cell that shows a lack of contact inhibition of growth *in vitro*, a benign tumor cell that is incapable of metastasis *in vivo*, or a cancer cell that is capable of metastases *in vivo* and that may recur after attempted removal. The term "tumorigenesis" is used to denote the induction of cell
10 proliferation that leads to the development of a neoplastic growth.

The terms "therapeutically effective amount" and "therapeutically effective period of time" are used to denote known treatments at dosages and for periods of time effective to reduce neoplastic cell growth. Preferably, such administration should be parenteral, oral, sublingual,
15 transdermal, topical, intranasal, or intrarectal. When administered systemically the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.1 μM to about 10 μM . For localized administration, much lower concentrations than this may be effective, and much higher concentrations
20 may be tolerated. One of skill in the art will appreciate that such therapeutic effect resulting in a lower effective concentration of the histone deacetylase inhibitor may vary considerably depending on the tissue, organ, or the particular animal or patient to be treated according to the invention.

25 In a preferred embodiment, the therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μM to about 20 μM . In a particularly preferred embodiment, the therapeutic composition

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is administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.05 μM to about 15 μM . In a more preferred embodiment, the blood level of antisense oligonucleotide is from about 0.1 μM to about 10 μM .

5 For localized administration, much lower concentrations than this may be therapeutically effective. Preferably, a total dosage of antisense oligonucleotide will range from about 0.1 mg to about 200 mg oligonucleotide per kg body weight per day. In a more preferred
10 embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 20 mg oligonucleotide per kg body weight per day. In a most preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 10 mg oligonucleotide per kg body weight per day. In a particularly preferred embodiment, the
15 therapeutically effective amount of a histone deacetylase antisense oligonucleotide is about 5 mg oligonucleotide per kg body weight per day.

 In certain preferred embodiments of the third aspect of the invention, the method further comprises administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically
20 effective period of time. In some preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide, as described *supra*.

 The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a
25 sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μM to about 10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule

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inhibitor from about $0.05\mu\text{M}$ to about $10\mu\text{M}$. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about $0.1\mu\text{M}$ to about $5\mu\text{M}$. For localized administration, much lower concentrations than this may be effective. Preferably, a total
5 dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred
10 embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective synergistic amount of histone deacetylase small molecule inhibitor (when administered with an antisense oligonucleotide)
15 is about 5 mg per kg body weight per day.

Certain preferred embodiments of this aspect of the invention result in an improved inhibitory effect, thereby reducing the therapeutically effective concentrations of either or both of the nucleic acid level inhibitor (*i.e.*, antisense oligonucleotide) and the protein level inhibitor (*i.e.*, histone
20 deacetylase small molecule inhibitor) required to obtain a given inhibitory effect as compared to those necessary when either is used individually.

Furthermore, one of skill will appreciate that the therapeutically effective synergistic amount of either the antisense oligonucleotide or the histone deacetylase inhibitor may be lowered or increased by fine tuning
25 and altering the amount of the other component. The invention therefore provides a method to tailor the administration/treatment to the particular exigencies specific to a given animal species or particular patient. Therapeutically effective ranges may be easily determined for example

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empirically by starting at relatively low amounts and by step-wise increments with concurrent evaluation of inhibition.

In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell

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differentiation comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time.

In certain embodiments where the agent of the first aspect of the invention is a histone deacetylase small molecule inhibitor, therapeutic compositions of the invention comprising said small molecule inhibitor(s) are administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μ M to about

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- 10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05 μM to about 10 μM . In a more preferred embodiment, the blood level of histone
- 5 deacetylase small molecule inhibitor is from about 0.1 μM to about 5 μM . For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total
- 10 dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day.
- 15 In a sixth aspect, the invention provides a method for investigating the role of a particular histone deacetylase isoform in cellular proliferation, including the proliferation of neoplastic cells. In this method, the cell type of interest is contacted with an amount of an antisense oligonucleotide that inhibits the expression of one or more specific histone deacetylase isoform,
- 20 as described for the first aspect according to the invention, resulting in inhibition of expression of the histone deacetylase isoform(s) in the cell. If the contacted cell with inhibited expression of the histone deacetylase isoform(s) also shows an inhibition in cell proliferation, then the histone deacetylase isoform(s) is required for the induction of cell proliferation. In
- 25 this scenario, if the contacted cell is a neoplastic cell, and the contacted neoplastic cell shows an inhibition of cell proliferation, then the histone deacetylase isoform whose expression was inhibited is a histone deacetylase isoform that is required for tumorigenesis. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2,

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HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

Thus, by identifying a particular histone deacetylase isoform that is
5 required for in the induction of cell proliferation, only that particular histone deacetylase isoform need be targeted with an antisense oligonucleotide to inhibit cell proliferation or induce differentiation. Consequently, a lower therapeutically effective dose of antisense oligonucleotide may be able to effectively inhibit cell proliferation.
10 Moreover, undesirable side effects of inhibiting all histone deacetylase isoforms may be avoided by specifically inhibiting the one (or more) histone deacetylase isoform(s) required for inducing cell proliferation.

As previously indicated, the agent of the first aspect includes, but is not limited to, oligonucleotides and small molecule inhibitors that inhibit
15 the activity of one or more, but less than all, HDAC isoforms. The measurement of the enzymatic activity of a histone deacetylase isoform can be achieved using known methodologies. For example, Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) describe the assessment of histone deacetylase enzymatic activity by the detection of acetylated histones in
20 trichostatin A treated cells. Taunton et al. (*Science* 272: 408-411, 1996) similarly describes methods to measure histone deacetylase enzymatic activity using endogenous and recombinant HDAC. Both Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) and Taunton et al. (*Science* 272: 408-411, 1996) are hereby incorporated by reference.

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Preferably, the histone deacetylase small molecule inhibitor(s) of the invention that inhibits a histone deacetylase isoform that is required for induction of cell proliferation is a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of fewer
5 than all histone deacetylase isoforms.

In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase
10 isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6,
15 HDAC-7, or HDAC-8.

The phrase "inducing cell differentiation" and similar terms are used to denote the ability of a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or combination thereof) to induce differentiation in a contacted cell as compared to a cell that is not
20 contacted. Thus, a neoplastic cell, when contacted with a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or both) of the invention, may be induced to differentiate, resulting in the production of a daughter cell that is phylogenetically more advanced than the contacted cell.

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In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two of the reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a
5 histone deacetylase small molecule inhibitor, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain preferred embodiments, each of
10 the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.

Antisense oligonucleotides that inhibit DNA methyltransferase are
15 described in Szyf and von Hofe, U.S. Patent No. 5,578,716, the entire contents of which are incorporated by reference. DNA methyltransferase small molecule inhibitors include, without limitation, 5-aza-2'-deoxycytidine (5-aza-dC), 5-fluoro-2'-deoxycytidine, 5-aza-cytidine (5-aza-C), or 5,6-dihydro-5-aza-cytidine.

20 In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In preferred embodiments, the cell
25 proliferation is neoplasia.

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For purposes of this aspect, it is unimportant how the specific HDAC isoform is inhibited. The present invention has provided the discovery that specific individual HDACs are involved in cell proliferation or differentiation, whereas others are not. As demonstrated in this
5 specification, this is true regardless of how the particular HDAC isoform(s) is/are inhibited.

By the term "modulating" proliferation or differentiation is meant altering by increasing or decreasing the relative amount of proliferation or differentiation when compared to a control cell not contacted with an agent
10 of the first aspect of the invention. Preferably, there is an increase or decrease of about 10% to 100%. More preferably, there is an increase or decrease of about 25% to 100%. Most preferably, there is an increase or decrease of about 50% to 100%. The term "about" is used herein to indicate a variance of as much as 20% over or below the stated numerical values.

15 In certain preferred embodiments, the histone deacetylase isoform is selected from HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1.

The following examples are intended to further illustrate certain
20 preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the
25 appended claims.

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EXAMPLES

Example 1

Synthesis and Identification of Antisense Oligonucleotides

5 Antisense (AS) and mismatch (MM) oligodeoxynucleotides (oligos) were designed to be directed against the 5'- or 3'-untranslated region (UTR) of the targeted gene. Oligos were synthesized with the phosphorothioate backbone and the 4X4 nucleotides 2'-O-methyl modification on an automated synthesizer and purified by preparative reverse-phase HPLC.

10 All oligos used were 20 base pairs in length.

 To identify antisense oligodeoxynucleotide (ODN) capable of inhibiting HDAC-1 expression in human cancer cells, eleven phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-1 gene (GenBank Accession No. U50079) were
15 initially screened in T24 cells at 100 nM. Cells were harvested after 24 hours of treatment, and HDAC-1 RNA expression was analyzed by Northern blot analysis. This screen identified HDAC-1 AS1 and AS2 as ODNs with antisense activity to human HDAC-1. HDAC-1 MM oligo was created as a control; compared to the antisense oligo, it has a 6-base
20 mismatch.

 Twenty-four phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-2 gene (GenBank Accession No. U31814) were screened as above. HDAC-2 AS was identified as an ODN with antisense activity to human HDAC-2. HDAC-2
25 MM was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

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Twenty-one phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-3 gene (GenBank Accession No. AF039703) were screened as above. HDAC-3 AS was identified as an ODN with antisense activity to human HDAC-3. HDAC-3
5 MM oligonucleotide was created as a control; compared to the antisense oligonucleotide, it contains a 6-base mismatch.

Seventeen phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-4 gene (GenBank Accession No. AB006626) were screened as above. HDAC-4 AS1 and AS2
10 were identified as ODNs with antisense activity to human HDAC-4. HDAC-4 MM1 and MM2 oligonucleotides were created as controls; compared to the antisense oligonucleotides, they each contain a 6-base mismatch.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-5
15 gene (GenBank Accession No. AF039691) were screened as above. HDAC-5 AS was identified as an ODN with antisense activity to human HDAC-5.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-6
20 gene (GenBank Accession No. AJ011972) were screened as above. HDAC-6 AS was identified as an ODN with antisense activity to human HDAC-6. HDAC-6 MM oligo was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

Eighteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-7
25 gene (GenBank Accession No. AF239243) were screened as above. HDAC-7 AS was identified as an ODN with antisense activity to human HDAC-7.

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Fourteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-8 gene (GenBank Accession No. AF230097) were screened as above. HDAC-8 AS was identified as an ODN with antisense activity to human HDAC-8.

5

Example 2 HDAC AS ODNs Specifically Inhibit Expression at the mRNA Level

In order to determine whether AS ODN treatment reduced HDAC expression at the mRNA level, human A549 cells were treated with 50 nM of antisense (AS) oligonucleotide directed against human HDAC-3 or its corresponding mismatch (MM) oligo for 48 hours, and A549 cells were treated with 50 nM or 100 nM of AS oligonucleotide directed against human HDAC-1, HDAC-2, HDAC-4, HDAC-5, HDAC-6 or HDAC-7 or the appropriate MM oligonucleotide (100 nM) for 24 hours.

Briefly, human A549 and/or T24 human bladder carcinoma cells were seeded in 10 cm tissue culture dishes one day prior to oligonucleotide treatment. The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were grown under the recommended culture conditions. Before the addition of the oligonucleotides, cells were washed with PBS (phosphate buffered saline). Next, lipofectin transfection reagent (GIBCO BRL Mississauga, Ontario, CA), at a concentration of 6.25 µg/ml, was added to serum free OPTIMEM medium (GIBCO BRL, Rockville, MD), which was then added to the cells. The oligonucleotides to be screened were then added directly to the cells (*i.e.*, one oligonucleotide per plate of cells). Mismatched oligonucleotides were used as controls. The same concentration of oligonucleotide (*e.g.*, 50 nM) was used per plate of cells for each oligonucleotide tested.

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Cells were harvested, and total RNAs were analyzed by Northern blot analysis. Briefly, total RNA was extracted using RNeasy miniprep columns (QIAGEN). Ten to twenty μ g of total RNA was run on a formaldehyde-containing 1% agarose gel with 0.5 M sodium phosphate (pH 7.0) as the buffer system. RNAs were then transferred to nitrocellulose membranes and hybridized with the indicated radiolabeled DNA probes. Autoradiography was performed using conventional procedures.

Figures 9A-9I present results of experiments conducted with HDAC-1 (Figure 9A), HDAC-2 (Figure 9B), HDAC-6 (Figure 9C), HDAC-3 (Figure 9D), HDAC-4 (Figures 9E and 9F), HDAC-5 (Figure 9G), HDAC-7 (Figure 9H), and HDAC-8 (Figure 9I) AS ODNs.

Treatment of cells with the respective HDAC AS ODN significantly inhibits the expression of the targeted HDAC mRNA in human A549 cells.

15

Example 3 HDAC OSDNs Inhibit HDAC Protein Expression

In order to determine whether treatment with HDAC OSDNs would inhibit HDAC protein expression, human A549 cancer cells were treated with 50 nM of paired antisense or its mismatch oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 48 hours. OSDN treatment conditions were as previously described.

Cells were lysed in buffer containing 1% Triton X-100, 0.5 % sodium deoxycholate, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, plus protease inhibitors. Total protein was quantified by the protein assay reagent from Bio-Rad (Hercules, CA). 100 μ g of total protein was analyzed by SDS-PAGE. Next, total protein was transferred onto a PVDF membrane and probed with various HDAC-specific primary antibodies. Rabbit anti-

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HDAC-1 (H-51), anti-HDAC-2 (H-54) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1:500 dilution. Rabbit anti-HDAC-3 antibody (Sigma, St. Louis, MO) was used at a dilution of 1:1000. Anti-HDAC-4 antibody was prepared as previously described (Wang, S.H. *et al.*, (1999) *Mol. Cell. Biol.* 19:7816-27), and was used at a dilution of 1:1000. Anti-HDAC-6 antibody was raised by immunizing rabbits with a GST fusion protein containing a fragment of HDAC-6 protein (amino acid #990 to #1216, GenBank Accession No. AAD29048). Rabbit antiserum was tested and found only to react specifically to the human HDAC-6 isoform. HDAC-6 antiserum was used at 1:500 dilution in Western blots to detect HDAC-6 in total cell lysates. Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ).

As shown in Figure 10A, the treatment of cells with HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 ODNs for 48 hours specifically inhibits the expression of the respective HDAC isotype protein. Figure 10B presents dose dependent response for the inhibited expression of HDAC-1 protein in cells treated with two HDAC-1 AS ODNs. As predicted, treatment of cells with the respective mismatch (MM) control oligonucleotide does not result in a significant decrease in HDAC-1 protein expression in the treated cells.

In order to demonstrate that the level of HDAC protein expression is an important factor in the cancer cell phenotype, experiments were done to determine the level of HDAC isotype expression in normal and cancer cells. Western blot analysis was performed as described above.

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The results are presented in Table 3 clearly demonstrate that HDAC-1, HDAC-2, HDAC-3, HDAC-4, and HDAC-6, isotype proteins are overexpressed in cancer cell lines.

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Table 3
Expression Level of HDAC Isotypes in Human
Normal and Cancer Cells

<u>States of Cell</u>	<u>Tissue Type</u>	<u>Cell Designation</u>	<u>HDAC-1</u>	<u>HDAC-2</u>	<u>HDAC-3</u>	<u>HDAC-4</u>	<u>HDAC-6</u>
Normal	Breast Epithelial	HMEC	-	+	++	+	+
Normal	Foreskin Fibroblasts	MRHF	-	+	+	++	+
Cancer	Bladder	T24	+++	++	+++	++	+++
Cancer	Lung	A549	++	+++	+++	+++	++
Cancer	Colon	SW48	+++	+++	+++	+++	+++
Cancer	Colon	HCT116	++++	+++	+++	++++	+++
Cancer	Colon	HT29	+++	+++	+++	+++	+++
Cancer	Colon	NCI-H446	++	++++	+++	++++	++
Cancer	Cervix	Hela	+++	++++	+++	+++	+++
Cancer	Prostate	DU145	+++	+++	+++	++++	+++
Cancer	Breast	MDA-MB-231	++	+++	+++	+++	++++
Cancer	Breast	MCF-7	+++	+++	+++	++	++
Cancer	Breast	T47D	+++	+++	+++	++	+++
Cancer	Kidney	293T	+++	++++	++++	++	++
Cancer	Leukemia	K562	+++	++++	++++	++++	++++
Cander	Leukemia	Jurkat T	+++	++	++++	++	++

(-): not detectable; (+): detectable; (++) : 2X over (+); (+++) : 5X over (+); (++++): 10X over (+)

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Example 4

Effect of HDAC Isotype Specific OSDNs on Cell Growth and Apoptosis

5 In order to determine the effect of HDAC OSDNs on cell growth and cell death through apoptosis, A549 or T24 cells, MDAMB231 cells, and HMEC cells (ATCC, Manassas, VA) were treated with HDAC OSDNs as previously described.

10 For the apoptosis study, cells were analyzed using the Cell Death Detection ELISA^{Plus} kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's directions. Typically, 10,000 cells were plated in 96-well tissue culture dishes for 2 hours before harvest and lysis. Each sample was analyzed in duplicate. ELISA reading was done using a MR700 plate reader (DYNEX Technology, Ashford, Middlesex, England) at
15 410 nm. The reference was set at 490 nm.

 For the cell growth analysis, human cancer or normal cells were treated with 50 nM of paired AS or MM oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 72 hours. Cells were harvested and cell numbers counted by trypan blue exclusion using a
20 hemocytometer. Percentage of inhibition was calculated as $(100 - \text{AS cell numbers} / \text{control cell numbers})\%$.

 Results of the study are shown in Figures 11-13, and in Table 4 and Table 5. Treatment of human cancer cells by HDAC-4 AS, and to a lesser extent, HDAC 1 AS, induces growth arrest and apoptosis of various human
25 cancer. The corresponding mismatches have no effect. The effects of HDAC-4 AS or HDAC-1 AS on growth inhibition and apoptosis are significantly reduced in human normal cells. In contrast to the effects of HDAC-4 or HDAC-1 AS oligos, treatment with human HDAC-3 and HDAC-6 OSDNs has no effect on cancer cell growth or apoptosis, and

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treatment with human HDAC-2 OSDN has a minimal effect on cancer cell growth inhibition. Since T24 cells are p53 null and A549 cells have functional p53 protein, this induction of apoptosis is independent of p53 activity.

5

Table 4
Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Growth Inhibition (AS vs. MM)

	<u>Cancer</u> <u>Cells</u>	<u>Normal</u> <u>Cells</u>		
	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	++(+)	+(+)	+/-	+/-
HDAC-2 AS	+(+)	+/-	-	+/-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	++	++	+/-
HDAC-6 AS	-	-	+/-	-

"-": no inhibition, "+": <50% inhibition, "++": 50-75% inhibition,

"+++": >75% inhibition

10

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Table 5

Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Apoptosis After 48 Hour Treatment

5

	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	+	-		-
HDAC-2 AS	-	-	-	-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	+	++	-
HDAC-6 AS	-	-	-	-
TSA (100ng/ml)	++	++	++	+

"-": ≤ 2 x fold over non-specific background; "+": 2-3X fold; "++": 3-5X fold;

"+++": 5-8X fold; "++++": 8X fold

Example 5

10 Inhibition of HDAC Isotypes Induces the Expression of Growth Regulatory Genes

In order to understand the mechanism of growth arrest and
apoptosis of cancer cells induced by HDAC-1 or HDAC-4 AS treatment,
15 RNase protection assays were used to analyze the mRNA expression of cell
growth regulators (p21 and *GADD45*) and proapoptotic gene *Bax*.

Briefly, human cancer A549 or T24 cells were treated with HDAC
isotype-specific antisense oligonucleotides (each 50 nM) for 48 hours. Total
RNAs were extracted and RNase protection assays were performed to
20 analyzed the mRNA expression level of p21 and *GADD45*. As a control,
A549 cells were treated by lipofectin with or without TSA (250 ng/ml)
treatment for 16 hours. These RNase protection assays were done

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according to the following procedure. Total RNA from cells was prepared using "RNeasy miniprep kit" from QIAGEN following the manufacturer's manual. Labeled probes used in the protection assays were synthesized using "hStress-1 multiple-probe template sets" from Pharmingen (San Diego, California, U.S.A.) according to the manufacturer's instructions. Protection procedures were performed using "RPA IITM Ribonuclease Protection Assay Kit" from Ambion, (Austin, Tx) following the manufacturer's instructions. Quantitation of the bands from autoradiograms was done by using CycloneTM Phosphor System (Packard Instruments Co. Inc., Meriden, CT). The results are shown in Figures 14, 15 and Table 6.

Table 6

Up-Regulation of p21, GADD45 and Bax After Cell
Treatment with Human HDAC Isotype-Specific Antisenses

	A549			T24		
	p21	GADD45	Bax	p21	GADD45	Bax
HDAC-1	1.7	5.0	0.8	2.4	3.4	0.9
HDAC-2	1.1	1.2	1.0	1.0	1.0	0.9
HDAC-3	0.7	0.9	1.0	0.9	1.0	1.0
HDAC-4	3.1	5.7	2.6	2.8	2.7	1.9
HDAC-6	1.0	1.0	1.0	1.0	0.8	1.1
TSA vs lipofectin	2.8	0.6	0.8			

Values indicate the fold induction of transcription as measured by RNase protection analysis for the respective AS vs. MM HDAC isotype-specific oligos.

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Results of the experiments are presented in Table 6. The inhibition of HDAC-4 in both A549 and T24 cancer cells dramatically up-regulates both p21 and *GADD45* expression. Inhibition of HDAC-1 by antisense oligonucleotides induces p21 expression but more greatly induces *GADD45* expression. Inhibition of HDAC-4, upregulates *Bax* expression in both A549 and T24 cells. The effect of HDAC-4 AS treatment (50 nM, 48 hrs) on p21 induction in A549 cells is comparable to that of TSA (0.3 to 0.8 uM, 16 hrs).

Experiments were also conducted to examine the affect of HDAC antisense oligonucleotides on HDAC protein expression. In A549 cells, treatment with HDAC-4 antisene oligonucleotides results in a dramatic increase in the level of p21 protein (Figure 15).

Example 6

Cyclin Gene Expression Is Repressed by HDAC-1 AS Treatment

Human cancer A549 cells were treated with AS1, AS2 or MM oligo directed human HDAC1 for 48 hours. Total cell lysates were harvested and analyzed by Western blot using antibodies against human HDAC1, cyclin B1, cyclin A and actin (all from Santa Cruz Biotechnology, Inc., Santa Cruz, California). AS1 or AS2 both repress expression of cyclin B1 and A. Downregulation of cyclin A and B1 expression by AS1 and AS2 correlates well with their ability to inhibit cancer cell growth. (Figure 16)

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Example 7

Inhibition of Growth in Soft Agar

5 1.3 g granulated agar (DIDFCO) was added to 100 ml deionized water and boiled in a microwave to sterilize. The boiled agar was held at 55°C until further use. Iscove's Modified Dulbecco's Medium (GIBCO/BRL), 100x Penicillin-Streptomycin-Glutamine (GIBCO/BRL) and fetal bovine serum (medicorp) were pre-warmed at 37°C. To 50 ml sterile
10 tubes was added 9 ml Iscove's medium, 2 ml fetal bovine serum and 0.2 ml 100x Pen-Strep-Gln. Then 9 ml 55°C 1.3% agar was added to each tube. The tube contents were mixed immediately, avoiding air bubbles, and 2.5 ml of the mixture was poured into each sterile 6 cm petri dish to form a polymerized bottom layer. Dishes with polymerized bottom layers were
15 then put in a CO2 incubator at 37°C until further use. In 50 ml sterile tubes were prewarmed at 37°C for each 4 cell lines/samples, 20 ml Iscove's medium, 0.4 ml 100x Pen-Strep-Gln and 8 ml fetal bovine serum. Cells were trypsinized and counted by trypan blue staining and 20,000 cells were aliquotted into a sterile 15 ml tube. To the tube was then added DMEM
20 with low glucose (GIBCO/BRL) + 10% fetal bovine serum + Pen-Strep-Gln to a final volume of 1 ml. To the prewarmed 37°C mix in the 50 ml tube was quickly added 8 ml 55°C 1.3% agar, which was then mixed well. Nine ml of this mixture was then aliquotted to each 1 ml cells in the 15 ml tube which is then mixed and 5 ml aliquotted onto the polymerized bottom
25 layer of the 6 cm culture plates and allowed to polymerize at room temperature. After polymerization, 2.5 ml bottom layer mix was gently added over the cell layer. Plates were wrapped up in foil paper and

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incubated in a CO₂ incubator at 37°C for three weeks, at which time colonies in agar are counted. The results are shown in Figure 17.

These results demonstrate that an antisense oligonucleotide complementary to HDAC-1 inhibits growth of A549 cells in soft agar, but
5 antisense oligonucleotides complementary to HDAC-2 or HDAC-6, or mismatch controls, do not.

Example 8

Inhibition of HDAC Isotypes by Small Molecules

10

In order to demonstrate the identification of HDAC small molecule inhibitors, HDAC small molecule inhibitors were screened in histone deacetylase enzyme assays using various human histone deacetylase isotypic enzymes (*i.e.*, HDAC-1, HDAC-3, HDAC-4 and HDAC-6). Cloned
15 recombinant human HDAC-1, HDAC-3 and HDAC-6 enzymes, which were tagged with the Flag epitope (Grozing, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 96:4868-4873 (1999)) in their C-termini, were produced by a baculovirus expression system in insect cells.

Flag-tagged human HDAC-4 enzyme was produced in human
20 embryonic kidney 293 cells after transformation by the calcium phosphate precipitation method. Briefly, 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and antibiotics. Plasmid DNA encoding Flag-tagged human HDAC-4 was precipitated by ethanol and resuspend in sterile water. DNA-calcium
25 precipitates, formed by mixing DNA, calcium chloride and 2XHEPES-buffered saline solution, were left on 293 cells for 12-16 hours. Cells were return to serum-contained DMEM medium and harvested at 48 hour post transfection for purification of Flag-tagged HDAC-4 enzyme.

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HDAC-1 and HDAC-6 were purified on a Q-Sepharose column, followed by an anti-Flag epitope affinity column. The other HDAC isotypes, HDAC-3 and HDAC-4, were purified directly on an anti-Flag affinity column.

5 For the deacetylase assay, 20,000 cpm of an [³H]-metabolically-labeled acetylated histone was used as a substrate. Histones were incubated with cloned recombinant human HDAC enzymes at 37⁰C. For the HDAC-1 assay, the incubation time was 10 minutes, and for the HDAC-3, HDAC-4 and HDAC-6 assays, the incubation time was 2 hours. All assay conditions were pre-determined
10 to be certain that each reaction was linear. Reactions were stopped by adding acetic acid (0.04 M, final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate, and the released [³H]-acetic acid was quantified by liquid scintillation counting. For the inhibition studies, HDAC enzyme was preincubated with test compounds for 30 minutes at 4⁰C prior to the
15 start of the enzymatic assay. IC₅₀ values for HDAC enzyme inhibitors were identified with dose response curves for each individual compound and, thereby, obtaining a value for the concentration of inhibitor that produced fifty percent of the maximal inhibition.

20

Example 9

Inhibition of HDAC Activity in Whole Cells by Small Molecules

T24 human bladder cancer cells (ATCC, Manassas, VA) growing in culture were incubated with test compounds for 16 hours. Histones were
25 extracted from the cells by standard procedures (see *e.g.* Yoshida *et al.*, *supra*) after the culture period. Twenty µg total core histone protein was loaded onto SDS/PAGE and transferred to nitrocellulose membranes, which were then reacted with polyclonal antibody specific for acetylated histone H-4 (Upstate Biotech Inc., Lake Placid, NY). Horse Radish
30 Peroxidase conjugated secondary antibody was used at a dilution of 1:5000

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to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ). After exposure to film, acetylated H-4 signal was quantitated by densitometry.

- 5 The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit histone deacetylation in whole cells.

Example 10

10 Inhibition of Cancer Cell Growth by HDAC Small Molecule Inhibitors

- Two thousand (2,000) human colon cancer HCT116 cells (ATCC, Manassas, VA) were used in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to quantitatively determine cell proliferation and cytotoxicity. Typically, HCT116 cells were plated into each well of the 96-well tissue culture plate and left overnight to attach to the plate. Compounds at various concentrations were added into the culture media (final DMSO concentration 1%) and incubated for 72 hours. MTT solution (obtained from Sigma as powder) was added and incubated with the cells for 4 hours at 37°C in incubator with 5% CO₂. During the incubation, viable cells convert MTT to a water-insoluble formazan dye. Solubilizing buffer (50% N,N-dimethylformamide, 20% SDS, pH 4.7) was added to cells and incubated for overnight at 37C in incubator with 5% CO₂. Solubilized dye was quantitated by colorimetric reading at 570 nM using a reference of 630 nM. Optical density values were converted to cell number values by comparison to a standard growth curve for each cell line. The concentration test compound that reduces the total cell number to 50% that of the control treatment, *i.e.*, 1% DMSO, is taken as the EC₅₀ value.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can affect cell proliferation.

5

Example 11

Inhibition by Small Molecules of Tumor Growth in a Mouse Model

Female BALB/c nude mice were obtained from Charles River Laboratories (Charles River, NY) and used at age 8-10 weeks. Human prostate tumor cells (DU145, 2×10^6) or human colon cancer cells (HCT116; 2×10^6) or small lung core A549 2×10^6 were injected subcutaneously in the animal's flank and allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times, then approximately 30 mg tumor fragments were implanted subcutaneously through a small surgical incision under general anaesthesia. Small molecule inhibitor administration by intraperitoneal or oral administration was initiated when the tumors reached a volume of 100 mm^3 . For intraperitoneal administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/day) were dissolved in 100% DMSO and administered daily intraperitoneally by injection. For oral administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/days) were dissolved in a solution containing 65% polyethylene glycol 400 (PEG 400 (Sigma-Aldridge, Mississauga, Ontario, CA, Catalogue No. P-3265), 5% ethanol, and 30% water. Tumor volumes were monitored twice weekly up to 20 days. Each experimental group contained at least 6-8 animals. Percentage inhibition was calculated using volume of tumor from vehicle-treated mice as controls.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit the growth of tumor cells *in vivo*.

5

Example 12

Upregulation of p21 Expression and Down regulation of Cyclin Gene Expression Following Treatment with Small Molecule Inhibitor

Sulfonamide aniline (compound 3, Table 2) is a small molecule
10 HDAC1 specific inhibitor. Human HCT116 cells were treated with escalating doses of compound 3 for 16 hours. Total cell lysates were harvested and expression of p21^{WAF1}, cyclin B1, cyclin A and actin was analyzed by Western blot. Ariti-p21^{WAF1} antibody was purchased from BD Transduction Laboratories (BD Pharmingen Canada, Missasagua, Ontario).
15 Compound 3 clearly upregulates expression of p21^{WAF1} and represses the expression of cyclin A and B1. The expression profile of these cell cycle regulators correlates well with the ability of compound 3 to inhibit HCT116 proliferation in MTT assays (see Table 2),

20

Example 13

Cell Cycle Arrest Induced by HDAC Small Molecule Inhibitors

Human cancer HCT116 cells were plated at 2×10^5 per 10-cm dish and were left to attach to the dish overnight in the incubator. Cells were
25 treated with small molecule inhibitors at various concentrations (1 μ M and 10 μ M, typically, dissolved in DMSO) for 16 hours. Cells were harvested by trypsinization and washed once in 1X PBS (phosphate buffered saline). The cells were resuspended in about 200ul 1X PBS and were fixed by slowly adding 1 ml 70% ethanol at -20° C and were left at least overnight at

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-20° C. Fixed cells were centrifuged at low speed (1,000 rpm) for 5 minutes, and the cell pellets were washed again with 1X PBS. Nucleic acids from fixed cells were incubated in a staining solution (0.1% (w/v) glucose in 1X PBS containing 50 ug/ml propidium iodide) (Sigma-Aldridge, Mississauga, Ontario, CA) and RNase A (final 100 units/ml, (Sigma-Aldridge, Mississauga, Ontario, CA) for at least 30 minutes in the dark at 25° C. DNA content was measured by using a fluorescence-activated cell sorter (FACS) machine. Treatment of cells with all HDAC small molecule inhibitors in Table 2 results in a significant accumulation of cancer cell in G2/M phase of the cell cycle and concomitantly reduce the accumulation of cancer cells in S phase of the cell cycle. The ratio of cells in G2/M phase vs. cells in the S phase was determined. The Effective concentration (EC) of a small molecule inhibitor to induce a (G2+M)/S ratio of 2.5 is calculated, as shown in Table 2.

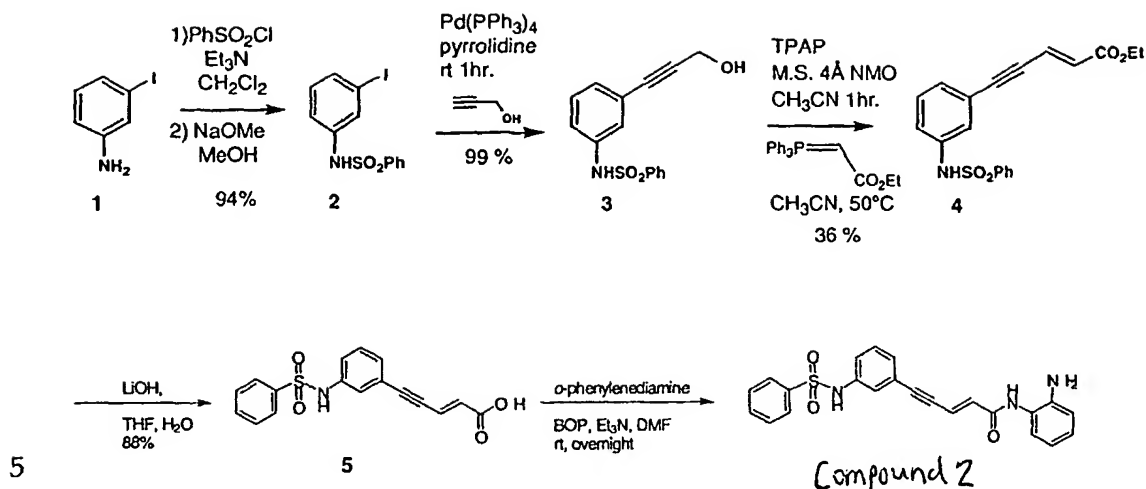
15

Example: 14

Synthesis of Small Molecule Compound No. 2

The following provides a synthesis scheme for small molecule Compound No. 2 from Table 2.

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Step 1: 3-(benzenesulfonylamino)-phenyl iodide (2)

To a solution of 3-iodoaniline (5 g, 22.8 mmol), in CH_2Cl_2 (100 mL),
 10 were added at room temperature Et_3N (6.97 mL) followed by
 benzenesulfonyl chloride (5.84 mL). The mixture was stirred 4 h then a
 white precipitate was formed. A saturated aqueous solution of NaHCO_3 ,
 was added and the phases were separated. The aqueous layer was
 extracted several times with CH_2Cl_2 and the combined extracts were dried
 15 over (MgSO_4) then evaporated. The crude mixture was dissolved in MeOH
 (100 mL) and NaOMe (6 g), was added and the mixture was heated 1 h at
 60°C . The solution became clear with time and HCl (1N) was added. The
 solvent was evaporated under reduced pressure then the aqueous phase
 was extracted several times with CH_2Cl_2 . The combined organic extracts
 20 were dried over (MgSO_4) and evaporated. The crude material was purified
 by flash chromatography using (100% CH_2Cl_2) as solvent yielding the title
 compound 21 (7.68g, 94 %) as yellow solid.

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¹H NMR: (300 MHz, CDCl₃): δ 7.82-7.78 (m, 2H), 7.60-7.55 (m, 1H), 7.50-7.42 (m, 4H), 7.10-7.06 (m, 1H), 6.96 (t, J = 8Hz, 1H), 6.87 (broad s, 1H).

Step 2: 3-(benzenesulfonylamino)-phenyl-propargylic alcohol (3)

To a solution of 2 (500 mg, 1.39 mmol) in pyrrolidine (5 mL) at room temperature was added Pd(PPh₃)₄ (80 mg, 0.069 mmol), followed by CuI (26 mg, 0.139 mmol). The mixture was stirred until complete dissolution. Propargylic alcohol (162 •L, 2.78 mmol) was added and stirred 6 h at room temperature. Then the solution was treated with a saturated aqueous solution of NH₄Cl and extracted several times with AcOEt. The combined organic extracts were dried over (MgSO₄) then evaporated. The residue was purified by flash chromatography using hexane/AcOEt (1:1) as solvent mixture yielding 3 (395 mg, 99 %) as yellow solid.

¹H NMR: (300 MHz, CDCl₃): δ 7.79-7.76 (m, 2H), 7.55-7.52 (m, 1H), 7.45 (t, J = 8Hz, 2H), 7.19-7.15 (m, 3H), 7.07-7.03 (m, 1H), 4.47 (s, 2H).

Step 3: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoate (4)

To a solution of 3 (2.75 g, 9.58 mmol) in CH₃CN (150 mL) at room temperature were added 4-methylmorpholine N-oxide (NMO, 1.68 g, 14.37 mmol) followed by tetrapropylammonium perruthenate (TPAP, 336 mg, .958 mmol). The mixture was stirred at room temperature 3 h, and then filtrated through a Celite pad with a fritted glass funnel. To the filtrate carbethoxymethylenetriphenyl-phosphorane (6.66 g, 19.16 mmol) was added and the resulting solution was stirred 3 h at room temperature. The solvent was evaporated and the residue was dissolved in CH₂Cl₂ and washed with a saturated aqueous solution of NH₄Cl. The aqueous layer was extracted several times with CH₂Cl₂, then the combined organic extract were dried over (MgSO₄) and evaporated. The crude material was purified

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by flash chromatography using hexane/AcOEt (1:1) as solvent mixture giving 4 (1.21 g, 36%) as yellow oil.

¹H NMR: (300 MHz, CDCl₃): δ 7.81 (d, J = 8Hz, 2H), 7.56-7.43 (m, 3H), 7.26-7.21 (m, 3H), 7.13-7.11 (m, 1H), 6.93 (d, J = 16 Hz, 1H), 6.29 (d, J = 16Hz, 1H), 4.24 (q, J = 7 Hz, 2H), 1.31 (t, J = 7Hz, 3H).

Step 4: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenic acid (5)

To a solution of 4 (888 mg, 2.50 mmol) in a solvent mixture of THF (10 mL) and water (10 mL) at room temperature was added LiOH (1.04 g, 25.01 mmol). The resulting mixture was heated 2 h at 60 °C and treated with HCl (1N) until pH 2. The phases were separated and the aqueous layer was extracted several times with AcOEt. The combined organic extracts were dried over (MgSO₄) then evaporated. The crude residue was purified by flash chromatography using CH₂Cl₂/MeOH (9:1) as solvent mixture yielding 5 (712 mg, 88 %), as white solid.

¹H NMR: (300 MHz, DMSO-*d*₆): δ 7.78-7.76 (m, 2H), 7.75-7.53 (m, 3H), 7.33-7.27 (m, 1H), 7.19-7.16 (m, 3H), 6.89 (d, J = 16 Hz, 1H), 6.33 (d, J = 16 Hz, 1H).

Step 5: Compound 2

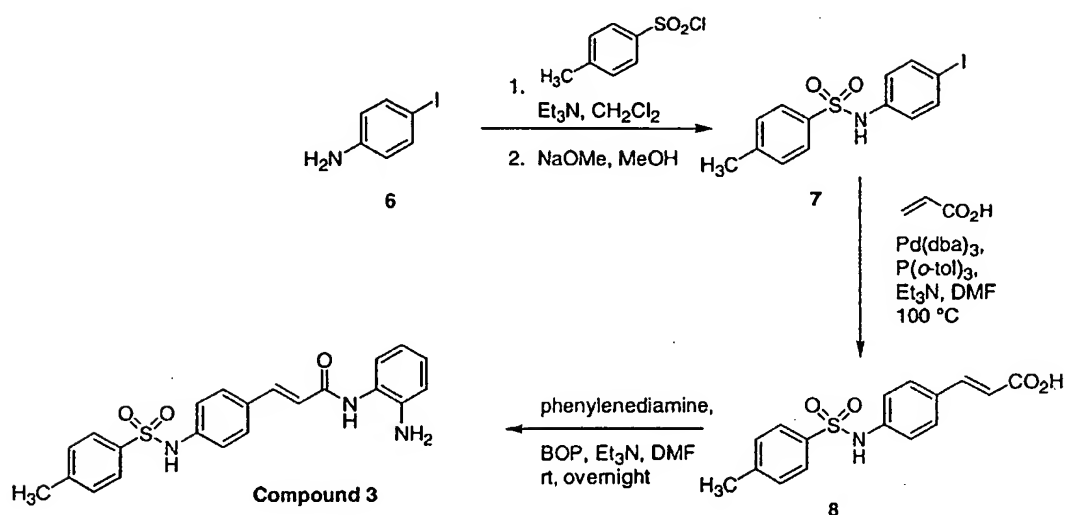
Coupling of 5 with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) afforded the anilide **Compound 2**.

¹H NMR: (300 MHz, DMSO *d*₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

¹³C NMR: (75 MHz, DMSO *d*₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 15
Synthesis of Small Molecule Compound No. 3

- 5 The following provides a synthesis scheme for Compound No. 3 from Table 2.



10 Step 1: 3-[4-(toluenesulfonylamino)-phenyl]-2-propenoic acid (8)

- To a solution of 7 (1.39 mmol), in DMF (10 mL) at room temperature were added tris(dibenzylideneacetone)dipalladium(0) ($\text{Pd}(\text{dba})_3$; 1.67 mmol), tri-*o*-tolylphosphine ($\text{P}(\text{o-tol})_3$, 0.83 mmol), Et_3N (3.48 mmol) and finally acrylic acid (1.67 mmol). The resulting solution was degassed and
- 15 purged several times with N_2 , then heated overnight at 100 °C. The solution was filtrated through a Celite pad with a fritted glass funnel then the filtrate was evaporated. The residue was purified by flash chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5) as solvent mixture yielding the title compound 8.

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Step 2: N-Hydroxy-3-[4-(benzenesulfonylamino)-phenyl]-2-propenamide(Compound 3)

The acid 8 was coupled with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium

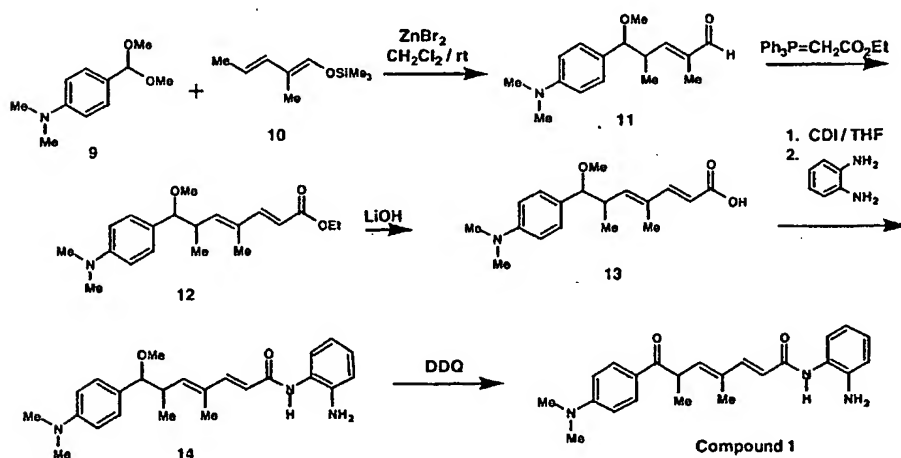
5 hexafluorophosphate (BOP) to afford the anilide **Compound 3**.

¹H NMR: (300 MHz, DMSO *d*₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

¹³C NMR: (75 MHz, DMSO *d*₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6;
10 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 16**Synthesis of Small Molecule No. Compound 1**

15 The following provides a synthesis scheme for small molecule Compound No. 1 from Table 2.



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Step 1: (11)

To a stirred solution of *p*-anisaldehyde dimethyl acetal (9) (10 mmol) in dry CH_2Cl_2 (60 mL) at rt was added 2-methyl-1-trimethylsilyloxypenta-1,3-diene (10) (*Tetrahedron*, 39: 881 (1983)) (10 mmol) followed by catalytic amount of anhydrous ZnBr_2 (25 mg). After being stirred for 5 h at rt, the reaction was quenched with water (20 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure.

10 Purification of the crude product by flash silica gel chromatography (25% ethyl acetate in hexane) afforded the desired aldehyde 11 in 68% yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CDCl_3) • 9.29 (s, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 6.29 (dq, J = 9.9, 1.2 Hz, 1H), 3.96 (d, J = 6.6 Hz, 1H), 3.20 (s, 3H), 3.05 (m, 1H), 2.94 (s, 6H), 1.60 (d, J = 0.9 Hz, 3H), 1.12 (d, J = 6.9 Hz, 3H).

15

Step 2: (12)

A mixture of aldehyde 11 (5.14 mmol) and ethyl (triphenylphosphoranylidene)acetate (2.15 g, 6.16 mmol) in toluene (25 mL) was heated at reflux overnight under N_2 . After removal of the solvent under reduced pressure, the crude product obtained was purified by flash silica gel chromatography (10% ethyl acetate in hexane) to give the title compound 12 in 96 % yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CDCl_3) δ 7.21 (dd, J = 15.6, 0.9 Hz, 1H), 7.06 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 5.69 (d, J = 15.6 Hz, 1H), 5.67 (br. d, J = 9.0 Hz, 1H), 4.17 (q, J = 7.2 Hz, 2H), 3.87 (d, J = 6.9 Hz, 1H), 3.18 (s, 3H), 2.93 (s, 6H), 2.81 (m, 1H), 1.59 (d, J = 1.2 Hz, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.05 (d, 6.6 Hz, 3H).

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Step 3: (13)

To a stirred solution of diene ester **12** (1.24 mmol) in methanol (10 mL) at rt was added aqueous LiOH 0.5 N solution (1.7mmol). After being stirred at 40 °C for 16 h, methanol was removed under reduced pressure and the resulting aqueous solution was acidified with 3N HCl (pH = ca. 4), extracted with ethyl acetate (25 × 3 mL), dried (MgSO₄), and concentrated under reduced pressure to give the desired carboxylic acid **13** in 98 % yield: **major isomer**: ¹H NMR (300 MHz, CD₃OD) δ 7.21 (d, J = 15.6, 0.6 Hz, 1H), 7.04 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H), 5.61 (d, J = 15.6 Hz, 1H), 5.60 (br. d, J = 10.0 Hz, 1H), 3.85 (d, J = 7.5 Hz, 1H), 3.13 (s, 3H), 2.87 (s, 6H), 2.81 (m, 1H), 1.52 (d, J = 1.5 Hz, 3H), 1.06 (d, J = 6.6 Hz, 3H).

Step 4: (14)

To a solution of carboxylic acid **13** (0.753 mmol) in anhydrous THF (10 mL) was added 1,1'-carbonyldiimidazole (0.790 mmol) at rt, and the mixture was stirred overnight. To the resulting solution was added 1,2-phenylenediamine (5.27 mmol), followed by trifluoroacetic acid (52 µl), and the reaction mixture was stirred for 16 h at rt. The reaction mixture was diluted with ethyl acetate (30 mL), washed with saturated NaHCO₃ solution (5 mL) and then water (10 mL), dried (MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in toluene) afforded the title compound **14** in 61% yield, as a mixture of two isomers in a ca.3 : 1 ratio: **major isomer**: ¹H NMR (300 MHz, CD₃OD) δ 7.28-7.02 (m, 5H), 6.79 (m, 2H), 6.68 (d, J = 8.7 Hz, 2H), 5.83 (d, J = 15.0 Hz, 1H), 5.69 (d, J = 9.6 Hz, 1H), 3.87 (d, J = 6.9 Hz, 1H), 3.19 (s, 3H), 2.94 (s, 6H), 2.80 (m, 1H), 1.61 (br. s, 3H), 1.07 (d, J = 6.6 Hz, 3H).

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Step 5: (Compound 1)

To a stirred solution of compound 14 (0.216 mmol) in wet benzene (2 mL, benzene : H₂O = 9 : 1) at room temperature was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.432 mmol). After being stirred
5 vigorously for 15 min., the mixture was diluted with ethyl acetate (30 mL), washed with water (2 × 5 mL), dried (anhydr.MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in hexanes, and then ethyl acetate only) afforded the title compound 35 (6 mg, 7% yield): ¹H NMR (300 MHz, CDCl₃) δ 7.83 (d, J = 9.0, 2H), 7.87 (br. s, 1H),
10 7.29 (d, J = 15.6 Hz, 1H), 7.27 (d, 7.8 Hz, 1H), 7.00 (m, 1H), 6.72 (m, 2H), 6.62 (d, J = 9.0 Hz, 2H), 5.97 (d, J = 15.6 Hz, 1H), 5.97 (d, J = 9.3 Hz, 1H), 4.34 (dq, J = 9.3, 6.9 Hz, 1H), 3.03 (s, 3H), 1.87 (br. s, 3H), 1.29 (d, J = 6.9 Hz, 3H); ¹³C
NMR (75 MHz, CDCl₃)
δ 12.6, 17.6, 39.9, 40.8, 110.7, 118.0, 119.0, 119.3, 123.8, 124.4, 125.1, 126.9,
15 130.6, 132.5, 140.8, 146.2, 153.4, 164.8, 198.6.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific
20 embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed is:

1. An agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms.
- 5 2. The agent according to claim 1, wherein the agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms, is an oligonucleotide.
- 10 3. The oligonucleotide according to claim 2, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA that encodes a portion of one or more histone deacetylase isoforms.
- 15 4. The oligonucleotide according to claim 3, wherein the oligonucleotide is a chimeric oligonucleotide.
- 20 5. The oligonucleotide according to claim 3, wherein the oligonucleotide is a hybrid oligonucleotide.
- 25 6. The oligonucleotide according to claim 3, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA selected from the group consisting of
 - (a) a nucleic acid molecule encoding a portion of HDAC-1 (SEQ ID NO:2),
 - (b) a nucleic acid molecule encoding a portion of HDAC-2 (SEQ ID NO:4),

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- (c) a nucleic acid molecule encoding a portion of HDAC-3 (SEQ ID NO:6),
- (d) a nucleic acid molecule encoding a portion of HDAC-4 (SEQ ID NO:8),
- 5 (e) a nucleic acid molecule encoding a portion of HDAC-5 (SEQ ID NO:10),
- (f) a nucleic acid molecule encoding a portion of HDAC-6 (SEQ ID NO:12),
- (g) a nucleic acid molecule encoding a portion of HDAC-7 (SEQ ID NO:14), and
- 10 (h) a nucleic acid molecule encoding a portion of HDAC-8 (SEQ ID NO:18).

7. The oligonucleotide according to claim 6 having a nucleotide
15 sequence of from about 13 to about 35 nucleotides.

8. The oligonucleotide according to claim 6 having a nucleotide
sequence of from about 15 to about 26 nucleotides.

20 9. The oligonucleotide according to claim 6 having one or more
phosphorothioate internucleoside linkage, being 20-26 nucleotides in
length, and being modified such that the terminal four nucleotides at the 5'
end of the oligonucleotide and the terminal four nucleotides at the 3' end of
the oligonucleotide each have 2'-O- methyl groups attached to their sugar
25 residues.

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10. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-1 (SEQ ID NO:2).

5 11. The oligonucleotide according to claim 10 that is SEQ ID NO:17 or SEQ ID NO:18.

12. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded
10 DNA encoding a portion of HDAC-2 (SEQ ID NO:4).

13. The oligonucleotide according to claim 12 that is SEQ ID NO:20.

15 14. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-3 (SEQ ID NO:6).

15. The oligonucleotide according to claim 14 that is SEQ ID
20 NO:22.

16. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-4 (SEQ ID NO:8).

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24. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-8 (SEQ ID NO:16).
- 5 25. The oligonucleotide according to claim 24 that is SEQ ID NO:32 or SEQ ID NO:33.
- 10 26. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the agent according to claim 1.
- 15 27. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the oligonucleotide according to claim 3.
- 20 28. The method according to claim 27, wherein cell proliferation is inhibited in the contacted cell.
- 25 29. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth retardation.
30. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth arrest.

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31. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo programmed cell death.

5 32. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo necrotic cell death.

33. The method according to claim 27, further comprising
10 contacting the cell with a histone deacetylase small molecule inhibitor.

34. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the
15 agent of claim 1.

35. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the
20 oligonucleotide of claim 3.

36. The method according to claim 35, wherein the animal is a human.

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37. The method according to claim 35, further comprising administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time.

5

38. A method for identifying a histone deacetylase isoform that is required for the induction of cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in the induction of cell proliferation indicates that the histone deacetylase isoform is required for the induction of cell proliferation.

10

39. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

15

40. A method for identifying a histone deacetylase isoform that is required for cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in cell proliferation indicates that the histone deacetylase isoform is required for cell proliferation.

20

41. The method according to claim 40, wherein the inhibitory agent is an oligonucleotide of claim 3.

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42. A method for identifying a histone deacetylase isoform that is required for the induction of cell differentiation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein an induction of cell differentiation indicates that the histone
5 deacetylase isoform is required for the induction of cell proliferation.

43. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

10 44. A method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a histone deacetylase small molecule inhibitor that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide
15 that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor.

45. A method for modulating cell proliferation or differentiation of a cell comprising inhibiting a specific HDAC isoform that is involved in
20 cell proliferation or differentiation by contacting the cell with an agent of claim 1.

46. The method according to claim 45, wherein the cell proliferation is neoplasia.

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47. The method according to claim 46, wherein the histone deacetylase isoform is selected from the group consisting of HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8.

5 48. The method according to claim 47, wherein the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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MAQTQGTRRKVCYYDGDVGNYYYGQGHMPKPHRIRMTHNLLN
YGLYRKMEIYRPHKANAEMTKYHSDDYIKFLRSIRPDNMSEYSKOMQRFNVGEDCPV
FDGLFEFCQLSTGGSVASAVKLNKQQTDI AVNWAGGLHHAKKSEASGFCYVNDIVLAI
LELLKYHQRVLYIDIDIHHGDGVEEAFYTTDRVMTVSFHKYGEYFPGTGLRDIGAGK
GKYAVYPLRDGIDDES YEAFKFPVMSKVMEFQPSAVVLQCGSDSLSGDRLGCFNL
TIKGHAKCVEFVKSFNLPMLMLGGGYTIRNVARCWTYETAVALDTEIPNELPYNDYF
EYFGPDFKLHISPSNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPEE
SGDEDEDDPKRISICSSDKRIACEEEFSDSEEEGEGGRKNSSNFKKAKRVKTEDEKE
KDPEEKKVTEEEKTKEEKPEAKGVKEEVKLA (SEQ ID NO:1)

FIG. 1A

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1 atgtctgtggg tcttgcccg ctggtgctgc tgtctcccac tgggtcatcc tgagaacaca
 61 gcctgagcgr ctctgtcact cggggtagac cagcggggga ggcgagcaag atggcgacaga
 121 cgcagggcac ccggaggaaa gtctgttact actacgacgg gcatgttggga aattactatt
 181 atggacaagg ccacccaatg aagcctcacc gaatccgcat gactcataat ttgctgctca
 241 actatggtct ctaccgaaa atggaatatct atcgccctca caaagccaat gctgaggaga
 301 tgaccaagta ccacagcgat gactacatta aattcttgcg ctccatccgt ccagataaca
 361 tgtcggagta cagcaagcag atgcagagat tcaacgttgg tgaggactgt ccagtattcg
 421 atggcctgtt tgagttctgt cagttgtcta ctggtggttc ctggcaagt gctgtgaaac
 481 ttaataagca gcagacggac atcgccgtga atgggctgg gggcctgcac catgcaaga
 541 agtccgaggc atctggcttc tgttacgtca atgatatcgt ctggccatc ctggaactgc
 601 taaagtatca ccagagggtg ctgtacattg acattgatat tcaccatggt gacggcgtgg
 661 aagaggcctt ctacaccag gaccgggtca tgaactgtgc ctttcataag tatggagagt
 721 acttcccagg aactggggac ctacgggata ccgggctgg caaagacaag tattatgctg
 781 ttaactaccc gctccgagac gggattgatg acgagtccta tgaggccatt ttcaagccgg
 841 tcatgtccaa agtaatggag atgttccagc ctagtgcggt tgccttacag gctggctcag
 901 actccctatc tggggatcgg ttaggttgct tcaatctatc tatcaaaagg cacgccaaat
 961 gtgtggaatt tgtcaagagc tttaacctgc ctatgctgat gctgggaggc ggtggttaca
 1021 ccattcgtaa cgttgcccgg tgctggacat atgagacagc tgtggccctg gatacggaga
 1081 tccctaataga gcttccatag aatgactact ttgaatactt tggaccagat ttcaagctcc
 1141 acatcagtc tccaatatg actaaccaga acacgaatga gtacctggag aagatcaaac
 1201 agcgaactgt tgagaaacct tgaaatgctgc cgcacgcacc tggggtccaa acgcaggcga
 1261 ttccctgagga cgccatccct gaggagagtg gcgatgagga cgaagacgac cctgacaagc
 1321 gcatctcgat ctgctcctct gacaaaacgaa ttgctgtga ggaagagtcc tccgattctg
 1381 aagaggaggg agaggggggc cgcaagaact ctccaactt caaaaaagcc aagagagtca
 1441 aaacagagga tgaaaaagag aaagacccag aggagaagaa aggaatcacc gaagaggaga
 1501 aaaccaaagg gagaaagcca gaagccaaag ggttcaagga gtagggccaag ttggcctgaa
 1561 tggacctctc cagctctggc ttctgtgta gtccctcagc tttctttccc c (SEQ ID NO:2)

FIG. 1B

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MAYSQGGGKKKCKVCYYDGDIGNYYYGQGHPMKPHRIRMTNLLL
NYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMHIPFNVGEDCP
AFDGLFEFCQLSTGGSVAGAVKLNRRQQTDMAVNWAGGLHHAKKYEASGFCYVNDIVLA
ILLELLKYHQRVLYIDIDIHHRGDGVVEEAFYTTDRVMTVSFYGEYFPGTGLRDIGAG
KGKYYAVNFPMCDGIDDESYGQIFKPIISKVMEYQPSAVVLQCGADSLSGDRLGCFN
LTVKGHAKCVEVVKTFNLPLMLGGGYTILRNVARCWTYETAVALDCEIPNELPYNDY
FEYFGPDFKLHISPSNMTNQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHE
DSGDEGEDPDKRISIRASDKRIACDEEFSDEGEGRNVADHKKGAKARIEED
KKETEDKKTDVKEEDKSKDNSGEKTDTKGTKSEQLSNP (SEQ ID NO:3)

FIG. 2A

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1 cgccgagctt tggcacctc cctgaccctc cccgcccggg taccgagcct tccggcgcc cctcctctc
 61 ctcccacggg cctgcccctc cccgcccggg tatcgccccc tatcgccccc acgtttccct cagccctttt
 121 ctctcccggc cgagccgagg gggcagcagc agcagcagca agcagcagc gcagcaggag caggcctttt
 181 gtggcgggcg tggccgggga gggcagcagc gggcagcagc gggcagcagc gggcagcagc gggcagcagc
 241 tgcctactac acgacgggtg tatggaaat ccataacttg agccactgac aataagaccca agattgtcca agattgtcca
 301 cctcatagaa tccgcatgac gggcccatat ttctacggtc atgttgagga gggctgagga gggctgagga gggctgagga
 361 gaaatatata tatatacaat ctctcaactg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 421 tatatacaat ctctcaactg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 481 catataatta atgttgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 541 ctctcaactg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 601 gctgttaatt gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 661 tacgttaatt gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 721 tatatcgata gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 781 cgtgtaata gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 841 agggatatgg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 901 atagacgatg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 961 tatcaacccta gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1021 ggttgtttca gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1081 aacttaccat gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1141 tggacatatg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1201 gattactttg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1261 aaccagaaca gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1321 atgttacctc gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1381 gacagtggag gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1441 aagcggatag gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1501 agaaatgttg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1561 gaaacagagg gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1621 gaaaaaacag gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1681 tctaccaaat gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1741 gaagacttct gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1801 actttttcgt gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1861 aaattttctt gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1921 gtcaaaaaaa gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga gggctgagga
 1981 aaaag (SEQ ID NO:4)

FIG. 2B

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MAKTVAIFYDPDVGNFHYGAGHPMKPHRLALTHSLVLHYGLYKK
MIVFKPYQASQHDMCRFHSEDIYIDFLQRVSPNTMQGFTKSLNAPNVGDDCPVFPGLFE
FCSRYTGASLQATQLNNKICDIANWAGGLHHAKKFEASGFCYVNDIVIGILELLLY
HPRVLYIDIDIHHGDGVQEAFLTDRVMTVSFHKYGNFYFFPGTGDMEVGAESGRYYC
LNVPLRDGIDDDQSYKHLFPVINQVDFYQPTCIVLQCGADSLGCDRLGCFNLSIRGH
CECVEYVKSFNIPPLLVLGGGGYTVRNVARCWTYETSLLLVEEAISEELPYSEYFEYFAP
DFTLHPDVSTRIENQSRQYLDQIRQTIFENLKMNLNHAPSVQIHVDVPADLLTYDRTDE
ADAEERGPEENYSRPEAPNEFYDGDHDNDKESDVEI (SEQ ID NO:5)

FIG. 3A

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FIG. 3B

1 ggaattcgcg ggcgcggcgg gcgcggggagg gcgcggggcct tgcgggggcct gctccgcgcg gaccatggc
 61 caagaccgtg gcctatttct acgaccccga cgtgggcaac cgtggggcctg tccactacg tccatggaca
 121 ccctatgaag ccccatcgcc atcgtcctca tggcatcgac agcctccaa ggctcgcctg catgacatgt agggcttcca
 181 taagaagatg atcgtcctca tccctgcagg acgtagggga tgcactgcca gcaaggagca accagctga acaacaagat
 241 ctccgaggac tatctgact atgccttca cgtacacag gcatctctt ggctggtgg acattgtgat tggcatcctg
 301 caagagtctt gttctgctcg gcatctctt ggctggtgg acattgtgat tggcatcctg ccataaatc cactggtgac
 361 gttctgctcg gcatctctt ggctggtgg acattgtgat tggcatcctg ccataaatc cactggtgac ccataaatc
 421 ctgtgatatt gcatctctt ggctggtgg acattgtgat tggcatcctg ccataaatc cactggtgac ccataaatc
 481 tggcttctgc tatgtcaacg tacattgaca cgggtcatga atgtatgaag ggcatctgatg ttctaccaac ttgggtgct
 541 tgggtgctc tacattgaca cgggtcatga atgtatgaag ggcatctgatg ttctaccaac ttgggtgctg ttcaatatc
 601 cctcactgac cgggtcatga atgtatgaag ggcatctgatg ttctaccaac ttgggtgctg ttcaatatc
 661 cacagggtgac atgtatgaag ggcatctgatg ttctaccaac ttgggtgctg ttcaatatc
 721 cctgcgggat ttctaccaac ttgggtgctg ttcaatatc ttgctggacat atcgagaatc ctgaagatgc
 781 ggtagtggac ttctaccaac ttgggtgctg ttcaatatc ttgctggacat atcgagaatc ctgaagatgc
 841 ctgtgatcga ttctaccaac ttgggtgctg ttcaatatc ttgctggacat atcgagaatc ctgaagatgc
 901 tgtcaagagc ttctaccaac ttgggtgctg ttcaatatc ttgctggacat atcgagaatc ctgaagatgc
 961 tgttgcctgc gcttccctat agtgaatact atcgagaatc ctgaagatgc agacccgcg ctttgaaaac
 1021 gcttccctat agtgaatact atcgagaatc ctgaagatgc agacccgcg ctttgaaaac agacctcctg
 1081 cagcaccgcg ctttgaaaac agacctcctg agacctatagc gaaagcgat gtagagattt aaggcttggg
 1141 ctttgaaaac agacctcctg agacctatagc gaaagcgat gtagagattt aaggcttggg aaggcttggg
 1201 agacctcctg agacctatagc gaaagcgat gtagagattt aaggcttggg aaggcttggg aaggcttggg
 1261 gaaagcgat gtagagattt aaggcttggg aaggcttggg aaggcttggg aaggcttggg aaggcttggg
 1321 gaaagcgat gtagagattt aaggcttggg aaggcttggg aaggcttggg aaggcttggg aaggcttggg
 1381 cactccttgg aaggcttggg aaggcttggg aaggcttggg aaggcttggg aaggcttggg aaggcttggg
 1441 ggggcttttg ctgactcttg ctgactcttg ctgactcttg ctgactcttg ctgactcttg ctgactcttg
 1501 cctgcttttc ctgactcttg ctgactcttg ctgactcttg ctgactcttg ctgactcttg ctgactcttg
 1561 caaggatagc tatctgggac tatctgggac tatctgggac tatctgggac tatctgggac tatctgggac
 1621 ttgcccctta ttcttccct ttgacttgcc ttgacttgcc ttgacttgcc ttgacttgcc ttgacttgcc
 1681 agacaaggac ttgacttgcc ttgacttgcc ttgacttgcc ttgacttgcc ttgacttgcc ttgacttgcc
 1741 ccttgcttcc aggaagatg aggaagatg aggaagatg aggaagatg aggaagatg aggaagatg
 1801 ctgaatccca gatgatggga gatgatggga gatgatggga gatgatggga gatgatggga gatgatggga
 1861 ctctcacttt tggctttatg tggctttatg tggctttatg tggctttatg tggctttatg tggctttatg
 1921 attttttgta cctttgatgg cctttgatgg cctttgatgg cctttgatgg cctttgatgg cctttgatgg

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MLAMKHQQELLEHQKLERHRQEQELEKQHRQKQLQQLKNKEKG
KESAVASTEVMKQLQEFVLNKKKALAHPNLNHCISCPRYWYGKTOHSSLDQSSPPQS
GVSTSYNHPVLGMYDAKDDFPLRKTASEP NLKLSRLKQKVAERRSSPLLRRKKGPPV
TALKKRPLDVTDSACSSAPGSGPSSPNNSSGSVAENGIAPAVPSIPAETSLAHRRLVA
REGSAAPLPLYTSPSLPNITLGLPATGPSAGTAGQQDTERLTLPALQQRSLSLFPGTHL
TPYLSTSPLERDGGAAHSPLLQHMVLLLEQPPAQAPLVTGLGALPLHAQSLVGADRVSP
SIHKLRLQHRPLGRTO SAPLPQNAQALQHLVIOQQHQQFLEKHKQOQQOQLQMNKIIP
KPEPARQPESHPEETEELREHQALLDEPYLDRLPQGQKEAHAQAGVQVKQEPESDE
ÆEAEPPREVEPGQRQPSQELLFRQQAALLLEQQRHQLRNYQASMEAAGIPVSFGGHR
PLSRAQSSPASATFPVSVQEPPTKPRFTTGLVYDTLMLKHQCTCGSSSSHPEHAGRIQ
SIWSRLQETGLRGKCECIRGRKATLEELQTVHSEAHTLLYG TNPLNRQKLD SKKLLGS
LASVFVRLPCGGVGVDSDTIWNEVHSAGAAARLAVGCVVELVFKVATGELKNGFAVVRP
PGHHAEESTPMGFCYFNSVAVAAKLLQQRLSVSKILLVDWDVHHGNGTQQAFYSDPSV
LYMSLHRYDDGNFFPGSGAPDEVGTGPGVGFNVMMAFTGGLDPPMGDAEYLA AFRTVV
MPIASEFAPDVVLASSGFDAVEGHPTPLGGYNLSARCFGYLTQLMGLAGGRIVLALAE
GGHDLTAICDASEACVSALLGNELDPLPEKVLQQRPNANAVRSMEKVMETHSKYWRCL
QRTTSTAGRSLIEAQTCENEEAETVTAMASLSVGVPKPAEKRPDEEPMEEEPPL (SEQ ID NO: 7)

FIG. 4A

1 ggagggttggtg gggccgcccgc cggcgagcac cgtccccgcc gccgcccagag ccgagagcccg
61 agcccgcgca ccgcccgcgc ccgcccgcgc cgcgcccga acagcctccc agcctgggccc
121 ccgggcgcg ccgtggccgc gtcgggctg tggccgcccgc agccgagcc cgcgcccgcg
181 cgggtggcgg cgcaggctga ggaatgccc aagcgagcc cggagcaggg ctagagcccg
241 ccgcccgcgc cgcgcccgcgc ggcgcccgcgc cgcgcccgcgc gccgcccgcgc catctgtccc
301 cgcgcccgcgc cgcgcccgcgc ggcgcccgcgc gccgcccgcgc cgcgcccgcgc tggagcccgc
361 cgggtccacac ccgcccgcgc ccgcccgcgc ggcgcccgcgc gccgcccgcgc gccgcccgcgc
421 gtgggacccc cgcgcccgcgc ccgcccgcgc ggcgcccgcgc gccgcccgcgc gccgcccgcgc
491 gaggcggcctt cgcgcccgcgc ccgcccgcgc ggcgcccgcgc gccgcccgcgc gccgcccgcgc
541 tctcccggtg cgcgcccgcgc ccgcccgcgc ggcgcccgcgc gccgcccgcgc gccgcccgcgc
601 tctgttcaac cgcgcccgcgc ccgcccgcgc ggcgcccgcgc gccgcccgcgc gccgcccgcgc
661 acgtctgtga ccagcccctc tctcgttccc caatgagctc tgaatcccgc ccgcccgcgc
721 cgttgaggc caatgagctc tgaatcccgc ccgcccgcgc ccgcccgcgc ccgcccgcgc
781 acattgctag caatgagctc tgaatcccgc ccgcccgcgc ccgcccgcgc ccgcccgcgc
841 gtggagctgc caatgagctc tgaatcccgc ccgcccgcgc ccgcccgcgc ccgcccgcgc
901 gcgctgcctc caatgagctc tgaatcccgc ccgcccgcgc ccgcccgcgc ccgcccgcgc
961 tctcactgc caatgagctc tgaatcccgc ccgcccgcgc ccgcccgcgc ccgcccgcgc

FIG. 4B-1

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1021 gcgctcaagc agaagcagca gatccagagg cagatcctca tcgccgagtt ccagaggcag
1081 cacgagcagc tctcccgga cagcagggcg cagctccacg agcacatcaa gcaataacag
1141 gagatgctgg ccatgaagca ccagcaggag ctgctggaac accagcggaa gctggagagg
1201 caccgccagg agcaggagct aggaagcagc caccgggagc caggcgtgca gcagctcaag
1261 aacaaggaga agggcaaaag agtgccgtg gccagcacag aagtgaagat gaagtataca
1321 gaatttgtcc tcaataaaaa gaaggcgtg gccaccgga atctgaacca ctgacttcc
1381 agagaccctc gctactggta cgggaaaacg cagcacagtt ccttgacca gaggttctcca
1441 cccagagcg gagtgtcgac ctctatgaac caccgggtcc tgggaatga cgacgccaaa
1501 gatgacttcc ctctaggaa aacagcttct gaaccgaatc tgaatcacg gtcaggcta
1561 aagcagaaag tggccgaaag acggagcagc cccctgttac gcaggaaaga cgggccagtg
1621 gtcaactgtc taaaaaagcg tccgttggat gtacagact ccgctgagc cagcgcacca
1681 ggctccggac ccagctcacc caacaacagc tccgggagcg tcgctggag gaaagggtatc
1741 gcgccgccg tcccagcat cccggcgag acgagtctgg cgcacagact tgtggcacga
1801 gaaggctcgg ccgctccact tcccctctac acatcgccat ccttgccaa catcacgctg
1861 ggcttgcctg ccaccggccc ctctgcggcg accgggagac agcaggactc cgagagactc
1921 acccttcccg cctccagca gaggctcttc ggggcagcgc acagccctct tctgcagcac
1981 ctgagcacct cgcccttggg gcggagcggg accggcacaa gcacaggcct tcacaggcct
2041 atggtcttac tggagcagcc accggcacaa gacccctcg gcagaggcct gggagtactg
2101 ccctccacg cacagtccct ggttggtgca gaccgggtgt cccctccat ccacaagctg
2161 cggcagcacc gccactggg gcggaccag tcggcccgcc cggccagaa cgccaggct
2221 ctgcagcacc tggatcata agcagcagcat gcagagtctc caggaaaca caagcagcag
2281 tccagcagc agcaactgca gatgaacaag atcatccca accaaagcga gccagcccg
2341 cagccggaga gccaccgga ggagacggag gaggagctcc gtgagcaca ggtctgtgctg
2401 gacgagccct acctggaccg gctgcccggg cagaaaggag cagacgcaca ggcggcgctg
2461 caggtgaagc aggagcccat tgagagcgat gaggaagagg cagagccccc acgggaggtg
2521 gagccgggccc agcgcagcc cagtgagcag gagctgctct tcagacagca agccctcctg
2581 ctggagcagc agcggatcca ccagctgagg aactaccagg cgtccatgga ggcggccggc
2641 atccccgtgt ccttcggcgg ccacaggcct ctgtcccggg cgagtcctc accgcgtct
2701 gccaccttcc ccgtgtccgt gcaggagccc cccaccaagc caggttccac gacaggcctc
2761 gtgtatgaca cgctgatgct gaagcaccag tgcacctgcg ggagtagcag cagccacccc
2821 gagcacgccg ggaggatcca gagcatctgg tcccgctg ccccgggggc cctccggggc

FIG. 4B-2

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2881 aaatgcgagt gcatccgcgg acgcaaggcc accctggaag agctacagac ggtgcactcg
 2941 gaaagccaca ccctcctgta tggcacgaac cccctcaacc ggcagaaact ggacagtaag
 3001 aaacttctag gctcgctcgc ctccgtgttc gtcgggtcc cttgcggtgg tgttggggtg
 3061 gacagtgaca ccataaggaa cgaggtgcac tcggcggggcagcccgcct ggctgtgggc
 3121 tgcgtggtag agctggtctt caaggtggcc acaggggagc tgaataatgg ctttgctgtg
 3181 gtccgcccc ctggacacca tgcggaggag agcacgccca tgggcttttg ctacttcaac
 3241 tccgcggccg tggcagccaa gcttctgcag cagaggttga gcgtgagcaa gatcctcatc
 3301 gtggactggg acgtgcacca tggaaacggg acccagcagg ctttctacag cgaccctagc
 3361 gtccctgtaca tgtccctcca ccgtacgac gatgggaaact tttccacag cagcggggct
 3421 cctgatgagg tgggcacagg gcccggtgtg ggtttcaacg tcaacacggc tttcacccgc
 3481 ggccctggacc ccccatggg agacgctgag tacttggcgg cttcagaaac ggtggtaatg
 3541 ccgatcgcca gcgagtttgc ccggatgtg gtgctggtgt catcaggctt cgatgccgtg
 3601 gagggccacc ccacccctct tgggggttac aacctctccg ccagatgctt cgggtacctg
 3661 acgaagcagg tgatggggctt ggctggcggc cggattgtcc tggccctcga gggaggccac
 3721 gacctgaccg ccatttgcga cgcttcggaa gcatgtgttt ctgccttgc tgggaaacgag
 3781 cttgatccct tccagaaaa tcatggagat ccacagcaag tactggcgtt gcctgcagc cacaacctcc
 3841 atggagaaag tcatggagat ccacagcaag tactggcgtt gcctgcagc cacaacctcc
 3901 acagcggggc gttctctgat cgaggctcag acttgcgaga acgaagaagc cgagacggtc
 3961 accgccatgg cctcgctgtc cgtggacgtg aagcccgccg aaaagagacc agatgaggag
 4021 cccatgggaag aggagccgcc cctgtagcac tccctcgaag ctgctgttct cttgtctgtc
 4081 tgtctctgtc ttgaagctca gccaaagaaac tttcccggtgt cagcctgctg tcccaccgtg
 4141 gggctctctt ggagcaccga gggacaccca gcgtgcaaca gccacgggaa gccttcttgc
 4201 cgcccaggcc cacaggcttc gagacgcaca tgcacgcctg ggcgtggcag cctcacaggg
 4261 aacacgggac agacgccggc gacgcgcaga cacacggaca cgcggaaagg aagcacactc
 4321 tggcgggtcc cgcaaggggc gccgtggaag aaaggagcct gtggcaacag gcggccgagc
 4381 tggcgaattc agttgacacg aggcacagaa acaaaaatct aaagatctaa taatacaaaa
 4441 caaacttgat taaaactggt gcttaaaagt tattaccac aactccacag tctctgtgta
 4501 aaccactcga ctcatcttgt agcttatatt ttttttaaa aggacgtttt ctacggctgt
 4561 gggccgcctc tgtgaaccat agcgggtgtgc ggcgggggggt ctgcaccggg gtgggggaca
 4621 gagggacctt taaagaaaa aaaactggac agaaacagga atgtagctg ggggagctgg
 4681 cttgagtttc tcaaaagcca tcggaagatg cgagtttgtg cctttttttt tattgctctg

FIG. 4B-3

4741 gtggattttt gtggctgggt tttctgaagt ctgaggaaca atgccttaag aaaaaacaaa
 4801 cagcaggaat cggctggaca gtttcctgtg gccagccgag cctggcagtg ctggcacccg
 4861 gagctggcct gacgcctcaa gacctgttgg cctgttttgg gttttaaagaa atcgggggcc
 4921 gcccgccggt cccgttttgg cctgttttgg gttttaaagaa atcgggggcc aggggctgca
 4981 agtggcaaat cccgttttgg cctgttttgg gttttaaagaa atcgggggcc gttccaataa
 5041 ctcacacgtc acatagcat acatagcat acatagcat acatagcat ccaaacgaat ccaaacgaat
 5101 ttgcagtggt acgacggaa ttgcagtggt acgacggaa ttgcagtggt acgacggaa ttgcagtggt
 5161 attttaggta taaataaata taaataaata taaataaata taaataaata taaataaata taaataaata
 5221 gaaacttact tgatttctat tgatttctat tgatttctat tgatttctat tgatttctat tgatttctat
 5281 gtatatatat atatatataa atatatataa atatatataa atatatataa atatatataa atatatataa
 5341 tgaatttggc ctcaagggtg ctcaagggtg ctcaagggtg ctcaagggtg ctcaagggtg ctcaagggtg
 5401 tcaagctcca gatgggttag gatgggttag gatgggttag gatgggttag gatgggttag gatgggttag
 5461 tacaagtttg tactttcatt tactttcatt tactttcatt tactttcatt tactttcatt tactttcatt
 5521 tcatgacat atgtacctaa atgtacctaa atgtacctaa atgtacctaa atgtacctaa atgtacctaa
 5561 ttgtatgaat tttttcacaa tttttcacaa tttttcacaa tttttcacaa tttttcacaa tttttcacaa
 5641 ttcctcacca ttttagcaat ttttagcaat ttttagcaat ttttagcaat ttttagcaat ttttagcaat
 5701 attcttggct gtacattttt gtacattttt gtacattttt gtacattttt gtacattttt gtacattttt
 5761 tttgtacgat gagttttctg gagttttctg gagttttctg gagttttctg gagttttctg gagttttctg
 5821 agtgagagga gggaccgtag gggaccgtag gggaccgtag gggaccgtag gggaccgtag gggaccgtag
 5881 ctgtccctagg agctgtataa agctgtataa agctgtataa agctgtataa agctgtataa agctgtataa
 5941 attacgaggg gtgggtgtgt gtgggtgtgt gtgggtgtgt gtgggtgtgt gtgggtgtgt gtgggtgtgt
 6001 gcccgccac cctgggaggg cctgggaggg cctgggaggg cctgggaggg cctgggaggg cctgggaggg
 6061 gtttaaaact ctctgaccac ctctgaccac ctctgaccac ctctgaccac ctctgaccac ctctgaccac
 6121 ttttgagcat gtcagcaatg gtcagcaatg gtcagcaatg gtcagcaatg gtcagcaatg gtcagcaatg
 6181 ccaactgcag cactgggcca cactgggcca cactgggcca cactgggcca cactgggcca cactgggcca
 6241 acccttgggt cctgggtgaac cctgggtgaac cctgggtgaac cctgggtgaac cctgggtgaac cctgggtgaac
 6301 ttactctttt ttctcttcaa ttctcttcaa ttctcttcaa ttctcttcaa ttctcttcaa ttctcttcaa
 6361 agcacatgaa gccaccagtt gccaccagtt gccaccagtt gccaccagtt gccaccagtt gccaccagtt
 6421 agttcagaca caccgtgctc caccgtgctc caccgtgctc caccgtgctc caccgtgctc caccgtgctc
 6481 tacagggttag cttctgaaat cttctgaaat cttctgaaat cttctgaaat cttctgaaat cttctgaaat
 6541 tcacttggtc actgggctgc actgggctgc actgggctgc actgggctgc actgggctgc actgggctgc

FIG. 4B-4

FIG. 4B-5

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6601 acggtcttgg gacttggttg actttcccct ccctggtggc cactcttgc tctgaagccc
6661 agattggcaa gaggagctgg tccattcccc attcatggca cagaacagtg gcagggccca
6721 gctagcaggc tcttctggcc tccctggcct cacttctctgc atagccctct ggggacccctg
6781 ccacctgccc tcttaccctg tcttaccctg ccgtggctta gctcagtgga tgcatactct cacttttttt
6841 ttttaagcag atgatgggat aacatggact aacatggact gctcagtgga caggttatca gtggggggac
6901 ttaattctaa tctcatcaca tctcatcaca atggagacga cctctgcaaa ggcctggcag ggggagggcaa
6961 gtttcatctg tcatcactcag gacacactcg gctcttctcc aatgtgctg agagcattac tgtgtagcct
7021 tttctttgaa gacacactcg gctcttctcc aacagcaagc acagcattac tccagggcag atggcagagg
7081 atctgcctcg gcgtctgcag gcgggaccac gtcagggagg gtccctcat gtgttctccc
7141 tgtgggtcct tggacctta gacctttct ccttttgcaa aggccttggg agccttggg ggcactggct
7201 gggagtcagc aagcgagcac tttatatccc tccgcctg tccagcgtg ccgacgtgc ccacgtgc
7261 cctcttggcg tctgacctg cctgcctg ctgccccga ggcgtggc tcaagtggt tcaagttttc
7321 cagccccac cagcaggcgg ggttctggag ggcactttg ggaaggacca atggactttg ttgctttct
7381 cccagcgtc cagggtct tttgcaagg gtttgcaagg agcactagca cacagccacg gctcatccc
7441 tttacttctt ttgaaaatct gtttgcaagg gtttgcaagg agcactagca cacagccacg gctcatccc
7501 aaagcaagtt tgatttttgc agcactagca gtttgcaagg gtttgcaagg agcactagca cacagccacg
7561 aacattcctt ctttactggt gtttactggt gtttactggt gtttactggt gtttactggt gtttactggt
7621 tgggcccacg tgttttatgg gtttttatgg gtttttatgg gtttttatgg gtttttatgg gtttttatgg
7681 gaatacattt ttttaagttt ttttaagttt ttttaagttt ttttaagttt ttttaagttt ttttaagttt
7741 ctttatattg tatacagatt tccctctca tgacatttgc agatacaaaa agtttgctc aaaggactac
7801 gcctttctgt tccctctca tgacatttgc agatacaaaa agtttgctc aaaggactac accaataata
7861 acaaaaacctt gaaggagagg agggcgggga agggcgggga agggcgggga agggcgggga agggcgggga
7921 aaattgtact ttttatgtta ttatctagca ctactcagaa ttacacagtga actgcctgtc ttttatgtg
7981 gaaaaaagt ttatctagca ctactcagaa ttacacagtga actgcctgtc ttttatgtg ttttatgtg
8041 gaaacagtgt ttagggaaa ctactcagaa ttacacagtga actgcctgtc ttttatgtg ttttatgtg
8101 tgatttggag gaattttgtt cctcactggc ctttgactgg ctttgactgg ctttgactgg ctttgactgg
8161 gccaggcgag cgcggcctc aaagagtccc cttttccgca cagctgtgtt gactttttaa ttacttttag
8221 ggcggactcg aaagagtccc cttttccgca cagctgtgtt gactttttaa ttacttttag ttacttttag
8281 gtgatgtatg gctaaagattt cactttaagc agtctgaaac tcttctattg taacgaagct gacgtgttc
8341 caattatact ttgcatcgaa aggaaccat tcttctattg taacgaagct gacgtgttc gacgtgttc
8401 ttagctcgcc ctcaacttgt ctctggcatt gattaaaagt ctgctattga aagaaaaag (SEQ ID NO:8)

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LRQGGTLTGKFMSTSSIPGCLLGVALEGDSPHGHASLLQHVL
LEQARQQSTLIAVPLHGQSPVLTGERVATSMRTVGKLP
RHRPLSRTQSSPLPQSPQAL
QQLVMQQQHQQFLEKQKQQQLQGLKILTKTGELPRQPT
THPEETEEELTEQQEVLLGE
GALTMPREGSTESESTQEDLEEEDEEEEDGEEEDCIQV
KDEEGESGAEEGPDLEEPGA
GYKKLFSDAQPLQPLQVYQAPLSLATVPHQALGR
TQSSPAAPGGMKSPDPQPVKHLFT
TGVVYDTFMLKKHQC
MCGNTHVHPEHAGRIQSIWSRLQETGLLSK
CERIRGRKATLDEI
QTVHSEYIHTLLYGTSP
LNRQKLDKSKLLGPISQKMYAVLPCGGIGVDS
DTVWNEMHSS
SAVRMAVGCLLELAFKVAAGELKNGFAIIRPPGH
AAEESTAMGFCFFNSVAITAKLLQ
QKLNVGKVLIVDWDIHHGNGTQQAFYNDPSVLYI
SLHRYDNGNFFPGSGAPEEVGGGP
GVGYNVNVAWTGGVDPPIGDVEYLTAFRTVVMPI
AHEFSPDVVTLVSAGFDAVEGHLSP
LGGYSVTARCFGLHTRQLMTLAGGRVVLAL
EGGHDLT
AICDASEACVSALLSVELQPL
DELVLQKPNINAVATLEKVIETQSKHWSCVQKFA
AGLGRSLREAQAGETEEAETVSA
MALLSVGAEQAAAAAREHSPRAEFPMEQEPAL (SEQ ID NO:9)

FIG. 5A

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```

1  ccctgcggca  gggtaggcacg  ctgaccggca  agttcatgag  cacatcctct  attcctggct
61  gcctgctggg  cgtggcactg  gagggcgacg  ggagccccc  cgggcatgcc  tccttgctgc
121  agcatgtgct  gttgctggag  caggcccgcc  agcagagcac  cctcattgct  gtgccactcc
181  acgggcagtc  cccactagt  cccactagt  gtgtggccac  cagcatgcgg  acggtaggca
241  agctcccgcg  gcatacgccc  ctgagccgca  ctcagtcctc  accgctgccg  cagagtcccc
301  aggccctgca  gcagctggtc  atgcaacaac  agcaccagca  gttcctggag  aagcagaagc
361  agcagcagct  acagctgggc  aagatcctca  ccaagacagg  ggagctgccc  aggcagccca
421  ccaccacccc  tgaggagaca  gaggaggagc  tgacggagca  gcaggaggtc  ttgctggggg
481  agggagccct  gaccatgccc  cgggagggtc  ccacagagag  tgagagcaca  caggaaagac
541  tggaggagga  ggacgaggaa  gaggatgggg  aggaggagga  ggattgcatc  caggttaagg
601  acgaggaggg  cgagagtgg  gctgaggagg  ggcccgaactt  ggaggagcct  ggtgctggat
661  acaaaaaact  gttctcagat  gccagccgc  tgacgccttt  gcagggtgtac  caggcgcccc
721  tcagccctggc  cactgtgccc  caccaggccc  tgggcccgtac  ccagtcctcc  cctgctgccc
781  ctggggggcat  gaagagcccc  ccagaccago  ccgtcaagca  cctcttcacc  acagggtgtg
841  tctacgacac  gttcatgcta  aagcaccagt  gcatgtgcgg  gaacacacac  gtgcaccctg

```

FIG. 5B-1

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901 agcatgctgg ccggtaccag agcatctggt cccggctgca ggagacaggc ctgcttagca
961 agtgcgagcg gatccgaggt cgcaaaagcca cgctagatga gatccagaca gtgcactctg
1021 aataccacac cctgctctac gggaccagtc ccctcaaccg gcagaagcta gacagcaaga
1081 agttgctcgg ccccatcagc cagaagatgt atgctgtgct gcccttgagg ggcacgagg
1141 tggacagtga caccgtgtgg aatgagatgc actcctccag tgctgtgctg atggcagtgg
1201 gctgcctgct ggagctggcc ttcaagggtgg ctgcaggaga gctcaagaat ggatttgcca
1261 tcattccggc ccaggacac caccgcgagg aatccacagc caccggattc tgcttcttca
1321 actctgtagc catcacggca aaactcctac agcagaagtt gaacgtgggc aaggctcctca
1381 tcgtggactg ggacattcac catggcaatg gcaccagca ggcgttctat aatgacccct
1441 ctgtgctcta catctctctg catcgctatg acaacgggaa cttctttcca ggctctgggg
1501 ctccctgaaga ggttggtgga ggaccaggcg tggggtacaa tgtgaacgtg gcatggacag
1561 gaggtgtgga ccccccatt ggagacgtgg agtaccttac agccttcagg acagtgggtga
1621 tgcccatgac ccacgagttc tcacctgatg tggtcctagt ctccgcccgg tttgatgctg
1681 ttgaaggaca tctgtctcct ctgggtgggt actctgtcac cgcagatgt tttggccact
1741 tgaccaggca gctgatgacc ctggcagggg gcccgggtggg gctggccctg gagggaggcc
1801 atgacttgac cgccatctgt gatgcctctg aggccttggt ctgggctctg ctcagtgtag
1861 agctgcagcc cttggatgag gcagtcttgc agcaaaagcc caacatcaac gcagtggcca
1921 cgctagagaa agtcatcgag atccagagca aacctggag ctgtgtgcag aagttcgccg
1981 ctggtctggg ccggtccctg cgagaggccc aagcaggtga ggccgaggag gccgagactg
2041 tgagcgccat ggccttgctg tcggtggggg ccgagcaggc ccaggctgctg gcagccccgg
2101 aacacagccc caggccggca gaggagccca tggagcagga gcctgcccctg tgacgccccg
2161 gcccccatcc ctctgggctt caccattgtg attttgttta ttttttctat taaaaacaaa
2221 aagtcacaca ttc (SEQ ID NO:10)

FIG. 5B-2

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1 mtstgqdstt trqrrsrqnp qspqqdssvt skrnikkjav prsipnlaev kkkgkmkklg
 61 gameedlivg lqgmdlnlea ealagtglvl deqlnefchl wddsfpegpe rlhaikqqli
 121 qeglldrcvs fgarfaekee lmlvhsleyi dlmettqymn egelrvladt ydsvylhpns
 181 yscaclasgs vlrlvdavlg aeirngmai i rppghhaqhs lmdgycmfhn vavaaryaqq
 241 khrirrvliv dwdvhhgqgt qftfdqdpv lyfsihryeq grfwphlkas nwsttgfgqg
 301 qgytinvpwn qvgmrdayi aafhlvllpv alefqpqlvl vaagfdalqg dpkgemaatp
 361 agfaqlthll mglaggklil sleggyaira laegvsaslh tllgdpcpm1 espgapcrsa
 421 qasvscalea lepfwevlvr stetverdnm eednveese eggweppvlp iltwplqsr
 481 tglvydqnmn nhcnlwdshh pevpqrilri morleelgia grcltltprp ateaelltch
 541 saeyvghlra tekmtrelh ressnfdisy icpstfacaq latgaacrly eavisgevin
 601 gaavvrppgh haeqdaacgf cffnsvavaa rhaqtisgha lrilivdwdv hhngngtqhmf
 661 eddpsvlyvs lhrydhgtff pmgdegassq igraagtgt vnvaungprm gdadylaawh
 721 rlvlpiafef npelvlvsag fdaargdplg gcqvspegya hlthllmgl a sgriiileg
 781 gynltsises maactrsilg dppllltlpr pplsgalasi tetiqvhray wrslrvmkve
 841 dregpssskl vtckapqpak prlaermtrr ekkvleagmg kvtsasfgee stpgqtnset
 901 avvalcqddp seaatggatl aqtiseaaig gamlgqttse eavggatpdq ttseetvgga
 961 ildqtseda vggatigqtt seeavggatl aqtiseaame gatldqttse eapggtelig
 1021 tplasstdhq tpptspvqgt tpqispstli gslrtlelgs esqgasesqa pgeenllgea
 1081 agggqdnadm lmqgsrgltd qafiyavtpl pwcphlvavc pipaagldvt qpcgdcgtiq
 1141 enwvclscyq vycgryingh mlqhhgnsgh plvlsyidl awcyycaqv hhqalldvkn
 1201 iahqnkfged mphph (SEQ ID:11)

FIG. 6A

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```
1  gggcagtccc  ctgaggagcg  gggctgggtg  aaacgctagg  ggcgggatct  ggcggagtgg
61  aagaaccgcg  gcaggggcca  agcctcctca  actatgacct  caaccggcca  ggattccacc
121  acaaccaggc  agcgaagaag  taggcagaac  cccagtcgc  cccctcagga  ctccagtgtc
181  acttcgaagc  gaaatattaa  aaaggagcc  gttcccgcct  ctatcccaa  tctagcggag
241  gtaagaaga  aaggcaaaat  gaagaagctc  ggccaagcaa  tggaagaaga  cctaatacgtg
301  ggaactgcaag  ggatggatct  gaacctcgag  gctgaagcac  tggcttgggtg  ggaaggccct
361  ttggatgagc  agttaaatga  attccattgc  ctctgggatg  acagcttccc  tcgctgcgtg
421  gagcgggctcc  atgccatcaa  ggagcaactg  atccaggagg  gcctcctaga  tggctgcgtg
481  tcctttcagg  cccggtttgc  tgaaaaggaa  gagctgatgt  tggttcacag  cctagaatat
```

FIG. 6B-1

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541 attgacctga tggaaacaac ccagtacatg aatgaggag aactccgtgt cctagcagac
 601 acccagact cagtttatct gcatacgaac tcatactcct gtgcctgcct ggcctcaggc
 661 tctgtccctca ggctgggtgga tgcggtccctg ggggctgaga tccggaacgg catggccatc
 721 attaggccctc ctggacatca cggccagcac agtcttatgg atggctattg catgttcaac
 781 cacgtggctg tggcagcccg ctatgctcaa cagaaacacc gcaccggag ggtccttacc
 841 gtagattggg atgtgcacca cggacaagga acacagttca ccttcgacca ggaacccagt
 901 gtccctctatt tctccatcca ccgctacgag cagggtaggt tctggcccca cctgaaggcc
 961 tctaactggt ccaccacagg ttctcggccaa gccaaggat ataccatcaa tgtgccttgg
 1021 aaccagggtg ggtgcggga tgcctgactac atgtgctt tcctgcacgt cctgctgcca
 1081 gtgcctctg agctccagcc tcagctggctc ctggtggccg ctggatttga tgccttgcaa
 1141 ggggacccca agggcgagat ggcggccact cggcaggggt tgcgccagt aaccacctg
 1201 ctcatgggtc tggcaggagg caagctgac ctgtctctgg aggtggcta caacctccgc
 1261 gccctggctg aaggcgtcag tgcctcgctc caccacctc tgggagaccc ttgcccctg
 1321 ccggagtcac ctggtgccc ctgcggagc gccaggctt cagtttcctg tgccttgga
 1381 gcccttgagc cctcttgga gttcttctg agatcaactg agaccgtgga gagggacaa
 1441 atggaggagg acaatgtaga ggagagcgag gaggaaggac cctgggagcc ccctgtgctc
 1501 ccaatccctga calggccagt gctacagtct cgcacagggc tggctctatga ccaaaatag
 1561 atgaatcact gcaacttgtg ggacagccac caccctgagg taccacagc catcttgccg
 1621 atcatgtgcc gtctggagg gctgggccc gctgggccc gccacacct gacacccgc
 1681 cctgcccacag aggtgagct gctcacctgt caccgtgctg agtacgtggg tcatctccgg
 1741 gccacagaga aatgaaaac ccgggagctg caccgtgaga gtccaactt tgaactccatc
 1801 tataatctgcc ccagtacctt cgcctgtgca cagcttgcca ctggcgctgc ctgcccctg
 1861 gtggaggctg tgccttcagg agaggtccctg aatgggtgctg ctgtgggtgcg tccccagga
 1921 caccacgcag agcaggatgc agcttgcgtt ttigtcttt tcaactctgt ggcgtgggt
 1981 gctcgccatg ccagactat cagtgggcat gacctacgga tccctgattgt ggattgggat
 2041 gtccaccacg gtaatggaac tcagcacatg ttgaggatg acccagtggt gctatatgtg
 2101 tccctgcacc gctatgatca tggcaccttc tccccatgg tcccatgagg tgcagcagc
 2161 cagatcggcc gggccgcggg cacaggcttc accgtcaacg tggcatggaa cgggccccgc
 2221 atgggtgatg ctgactacct agctgcctgg catcgccctgg tgcctcccat tgcctacgag
 2281 ttaaacccag aactgggtgct ggtctcagct ggctttgatg ctgcacgggg ggatccgctg

FIG. 6B-2

2341 gggggctgcc aggtgtcacc tgagggttat gccaccta cccacctgct gatgggcctt
 2401 gccagtggcc gcattatcct tatcctagag ggtggctata acctgacatc catctcagag
 2461 tccatggctg cctgcactcg ctccctcctt ttccctcctt cacccttgct gaccctgcca
 2521 cggccccccac tatcaggggc cctggcctca atcactgaga ccatccaagt ccactgcaga
 2581 tactggcgca gcttacgggt catgaaggca gaagacagag aaggaccctc cagttctaag
 2641 ttggtcacca agaaggcacc ccaaccagcc aaacctaggt tagctgagcg gatgaccaca
 2701 cgagaaaaga aggttctgga agcaggcatg gggaagtca cctcgcatc atttgggaa
 2761 gagtccactc caggccagac taactcagag taactcagag acagctgtgg tggccctcac tcaggaccag
 2821 ccctcagagg cagccacagg gggagccact gggcccaga ctggccctga ccatttctga ggcaggccatt
 2881 gggggagcca tgctgggcca gaccacctca gaggaggctg gcgggggagc cactccggac
 2941 cagaccacct cagaggagac tgtgggagga gccattctgg accagaccac ctcagaggat
 3001 gctgttgggg gagccacgct gggccagact acctcagagg aggcgttagg aggagctaca
 3061 ctggcccaga ccatctcgga ggcagccatg gagggagcca cactggacca cactacgtca
 3121 gaggaggctc cagggggcac cgagctgac cgaaactctc tagcctcgag cacagaccac
 3181 cagaccccc caacctcacc tgtgcaggga actacacccc agatatctcc cagtacactg
 3241 attgggagtc tcaggacctt ggagctaggg agcgaacctc agggggcctc agaactctcag
 3301 gcccaggag aggagaacct accaggagag gcagctggag gtcaggacat ggctgattcg
 3361 atgctgacgc agggatctag gggcctcact gatcaggcca tattttatgc tgtgacacca
 3421 ctgccctggt gtcccattc ggtggcagta tgcccatac ctgcagcagg cctagacgtg
 3481 acccaacctt gtggggactg tggaacaatc caagagaact gggtgtgtct ctcttgctat
 3541 caggtctacc gtggtcgtta catcaatggc cacatgctcc aacaccatgg aaattctgga
 3601 caccgcctgg tcctcagcca catcgacctg tcagcctggc gttactactg tcaggccctat
 3661 gtccaccacc aggcctctct agatgtgaag aacatcgccc accagaaaca gtgtggggag
 3721 gatagcccc acccacata agcccagaa tacggtccct ctccaccttc tgaggcccac
 3781 gatagaccag ttccagcctg ttccaggctg taccttggat gaggggtagc ctccactgc
 3841 atcccatcct gaatatcctt tgcaactccc caagagtgtct tatttaagtg ttaatacttt
 3901 taagagaact gcgacgatta attgtggatc tccccctgcc catcgcccgc ttgaggggca
 3961 cactactcc agccagaaag gaaagggggg cagctcagtg gcccagaag ggagccgata
 4021 tcatgaggat aacattggcg ggaggggagt taactggcag gcatggcaag gttgcatatg
 4081 taataaagta caagctgtt (SEQ ID NO: 12)

FIG. 6B-3

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1 mdlrvqgrpp vepppeptll alqrpqrlhh hlflaglqqq rsvepmlsm dtpmpelqv9
 61 pgeqelrqll hkdkskrsav assvvkqla evilkkqaa lertvhpnsip gipyrtlepi
 121 etegatrsm1 ssflppvpsi psdppehfpl rktvsepnlk lrykpkksle rrknpllrke
 181 sappslrrrp aetlgdssps ssstpasgcs spndsehgnp pilgdsdrrt hptlgprgpi
 241 lgsphtplfl phglepeagg clpsrlqpil lldpsgshap lltvpglgpl pfhfaqsimt
 301 terlsgsglh wplsrttrsep lppsatappp pppmqprleq lkthvqviki sakpsekpri
 361 rqipseaedle tdgggpgqv ddglehrelg hgqpeargpa plqghpqvii weqqlagrl
 421 prgstgdcvi lplaaggghrp lsraqsspaa pasisapepa sqarvlssse tpartlpflt
 481 gliydsvm1k hqscgdnr hpehagriqs iwsrlqergl rsqceclrgk kasieelqsv
 541 hserhvlllyg tnplsr1kld ngklagiliaq rmfemlpcgg vgvdttdtiwn elhssnaarw
 601 aagsvtdlaf kvasrelkng favvrppghh adhstamgfc ffnsvaiacr qlqqqskask
 661 askilivdwd vhhngntqqt fyqdpvlyi slhrhddgnf fpgsgavdev gagsgegfnv
 721 nvawaggldp pmgdpeylaa frivvmpiar efspdlvls agfdaaeghp aplggyhvsa
 781 kcfgymtqq1 mnlaggavvl alegghdlt a icdaseacva allgnrvdpl seegwkqkpq
 841 pqchplsgr dpgaq (SEQ ID NO:13)

FIG. 7A

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1 ataataaccta ccttgccagga ccacgacagg ataatgtgag gaaaaacccc catgagagtg
61 ttttgccatt gtcaagttag cctgaggagg gctgaggggg gatcaggctg tatcatgccc
121 ccgaggacaa actttccagt ttaccctgct tacccttctt gtccttaggc tgcccaggcc
181 cctgcccaga cacaccaggc cctcagccgc agcccatgga cctgcgggtg ggccagcggc
241 cccagtgga gcccaccca gagccacat tgctggcct gcagcgtccc cagcgcctgc
301 accaccacct cttcctagca ggcctgcagc agcagcgtc ggtggagccc atgaggctct
361 ccattggacac gccgacgcc gagttagcagg tggacccca agaacagag ctgcggcagc
421 ttctccacaa ggacaagagc aagcgaagtg ctgtagccag cagcgtggtc aagcagaagc
481 tagcggagggt gattctgaaa aaacagcagg cggccctaga aagaacagtc catcccaaca
541 gccccggcat tccctacaga accccggagc ccctggagac ggaaggagcc accgctcca
601 tgctcagcag ccttccgcct cctgctcca gccgcccag tgacccccc gagcactccc
661 ctctgcgcaa gacagtctct gagcccaacc tgaagctgcg ccataagccc aagaagtccc
721 cggagcggag gaagaatcca ctgctccgaa aggagagtgc gccccccagc cccggcggc
781 ggcccgcaga gaccctcggg gactcctccc caagtagtag cagcacgccc gcatcagggt
841 gcagtccccc caatgacagc gagcacggcc ccaatcccat cctgggagcag agtgaccgca
901 ggacccatcc gactctggg cccggggggc caatcctggg gagcccccac actcccctct
961 tectgcccc tggcttggag cccgaggctg ggggcacctt gccctcccgc ctgcagccca
1021 ttccctctcct ggacccctca ggctctcatg cccgcttgcct gactgtgccc gggcttgggc
1081 ccttgccctt ccactttgcc cagtccctaa tgaccaccga gcggctctct gggtcaggcc
1141 tccactggcc actgagccgg actcgtcag agcccttgc cccagtgcc accgctccc
1201 caccgcccgg ccccatgcag cccgcctgg agcagctcaa aactcacgtc caggtgatca
1261 agaggtcagc caagccgagt gagaagcccc ggctgcggca gataccctcg gctgaagacc
1321 tggagacaga tggcggggga ccggggccagg tggtaggacga cggcccgagg cacagggagc

FIG. 7B-1

1381 tggggccatgg gcagcccgag gccagaggcc ccgctcctct ccagcagcac ctcaggtgt
 1441 tgctctggga acagcagcga ctggctgggc ggctccccc ggagcagcac ggggacactg
 1501 tgctgcttcc tctggcccag ggtgggcacc ggctctgtc ccgggctcag tcttcccag
 1561 ccgcacctgc ctcactgtca gcccagagc ctgccagcca ctgccagtc ctctccagct
 1621 cagagacccc tgccaggacc ctgcccttca ccacagggct gatctatgac tcggtcatgc
 1681 tgaagcacca gtgctcctgc ggtgacaaca gcaggcacc ggagcacgcc ggcgcacccc
 1741 agagcatctg gtcccggctg caggagcggg ggctcggag ccagtgtgag tgtctccgag
 1801 gccggaaggc ctccctggaa gagctgcagt cggctccactc tgagcggcac gtgctcctct
 1861 acggcaccaa ccgctcagc cgcctcaaac tggacaacgg gaagctggca gggctcctgg
 1921 cacagcggat gtttagatg ctgccctgtg gtggggttgg ggtggacact gacaccatct
 1981 ggaatgagct tcatccccc aatgcagccc gctgggccc gtcagtgctc tctgacctcg
 2041 ctttcaaagt ggcttctcgt gagctaaaga atgggttcgc tgtggtgcgg ccccaggac
 2101 accatgcaga tcatcaaca gccatgggct tctgcttctt caactcagt gccatcgct
 2161 gccggcagct gcaacagcag agcaaggcca gcaaggccag caagatcctc attgtagact
 2221 gggacgtgca ccatggcaac ggcacccagc aaaccttcta ccaagacccc agtgtgctct
 2281 acatctccct gcatcgccat gacgacggca acttcttccc gggagtgagg gctgtggatg
 2341 aggtaggggc tggcagcggc gagggcttca atgtcaatgt ggcctgggct ggaggtcttg
 2401 acccccccat gggggatcct gagtacctgg ctgctttcag gatagtcgtg acgcccacg
 2461 ccgagagatt ctctccagac ctagtccctgg tgtctgccgg atttgatgct gctgagggtc
 2521 acccggtccc acLgggtggc taccatgttt ctgccaaatg ttttggatc atgacgcagc
 2581 aactgatgaa cctggcagga ggcgcagtgg tgctggcctt ggaggggtggc catgacctca
 2641 cagccatctg tgacgcctct gaggcctgtg tggctgctct tctgggtaac aggggtggatc
 2701 ccctttcaga agaaggctgg aaacagaaac cccaacctca atgccactcg ctctctggag
 2761 gccgtgatcc ggtgacacag taaatactgg ggctgcatgc agcgcctggc ctccctgtcca
 2821 gactcctggg tgcctagagt gccaggggct gacaaagaag aagtggaggc agtgaccgca
 2881 ctggcgctccc tctctgtggg catcctggct gaagataggg cctcggagca gctgtgtggag
 2941 gaggaagaac ctatgaatct ctaaggctct ggaaccatct gccgcccac catgcccttg
 3001 ggacctgggt ctcttctaac ccctggcaat agcccccat cctgggtctt tagagatcct
 3061 gtgggcaagt agttggaacc agagaacagc ctgctgctt tgacagttat ccaggggagc
 3121 gtgagaaat c (SEQ ID NO:14)

FIG. 7B-2

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1 meepeepads gqslvpvviy speyvsmcde lakipkrasm vhsleayal hkqmrivkpk
 61 vasmeematf htdaylqhlq kvsqegdddh pdsieyglgy dcpategifd yaaaiggati
 121 taaqclidgm ckvainwsg whhakkdeas gfcylndavl gilrlrrkfe rilyvdldlh
 181 hgdgvedafs ftskvmtvsl hkfsgffpg tgdvsdvglg kgryysvnpv iqdgigdeky
 241 yqicesvlke vyqafnpkav vlqlgadtia gdpmcfsfnt pvgigkclky ilqqlatli
 301 lggggynlan tarcwtyltg vilgkltisse ipdbefftay gpdylvletp scrpdrneph
 361 riqqilnyik gnlkhvv (SEQ ID NO:15)

FIG. 8A

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```

1  gaaattcggc acgagctcgt gccgaattcg gcacgagaac ggttttaagc ggaagatgga
61  ggagccggag gaaccggcgg acagtgggca gtcgctggtc ccggtttata tctatagtc
121 cgagtatgtc agtatgtgtg actccctggc caagatccc aaacgggcca gtatggtgca
181 ttcttttgatt gaagcatatg cactgcataa gcaaatgagg atagttaagc ctaagtggc
241 ctccatggag gagatggcca cctccacac tgatgcttat ctgcagcatc tccagaaggt
301 cagccaagag ggcgatgatg atcatccgga ctccatagaa tatgggctag gttatgactg
361 ccagccact gaaggatat ctgactatgc agcagctata ggaggggcta cgatcacagc
421 tgcccaatgc ctgattgacg gaatgtgcaa agtagcaatc aactggtctg gaggtggca
481 tcatgcaaaag aaagatgaag catctggttt tcgttatctc aatgatgctg tcctgggaat
541 attacgattg cgacggaaat ttgagcgtat tccctacgtg gattcggatc tgcaccatgg
601 agatggtgta gaagacgcat tcagtttcac ctccaaagtc atgaccgtgt ccctgcacaa
661 attctcccca ggatttttcc caggaacagg tgacgtgtcc gacgttggcc tagggaagg
721 acggtactac agtgtaaatg tgcccatcca ggatggcata caagatgaaa aatatacca
781 gatctgcgaa agtgtaactaa aggaagtata ccaagccttt aatcccaag cagtgggtctt
841 acagctggga gccgacacaa tagctgggga tcccatgtgc tcctttaaca tgcactccagt
901 gggaattggc aagtgtctca agtacatccc tcaatggcag ttggcaacac tcatttcggg
961 aggaggaggc tataaccttg ccaacacggc tcgatgctgg acatacttga ccggggtcat
1021 cctagggaaa acactatcct ctgagatccc agatcatgag tttttcacag catatggtcc
1081 tgatttatgtg ctggaaatca cgccaagctg ccggccagac cgcaatgagc ccaccgaat
1141 ccaacaaatc ctcaactaca tcaaaaggaa tctgaagcat gtggtctagt tgacagaaag
1201 agatcaggtt tccagagctg aggagtgggtg cctataatga agacagcgtg ttatgcaag
1261 cagtttgrgg aatttgtgac tgcaaggaaa attgaaaga aattacttcc tgaaaaattc
1321 caaaggggcat caagtggcag ctggcttcct ggggtgaaga ggcaggcacc ccagagtcct
1381 caactggacc taggggaaga aggagatarc ccacatttaa agttcttatt taaaaaaca
1441 cacacacaca aatgaaattt ttaatctttg aaaattattt ttaagcgaat tggggagggg
1501 agtattttaa tcatctttaa tgaaacagat cagaagctgg atgagagcag tcaccagttt
1561 gtagggcagg aggcagctga caggcagggn tngggcctcn ggaccancca ngtggagccc
1621 tgggagagan ggtactgac ngcagactgg gagg (SEQ ID NO:16)

```

FIG. 8B

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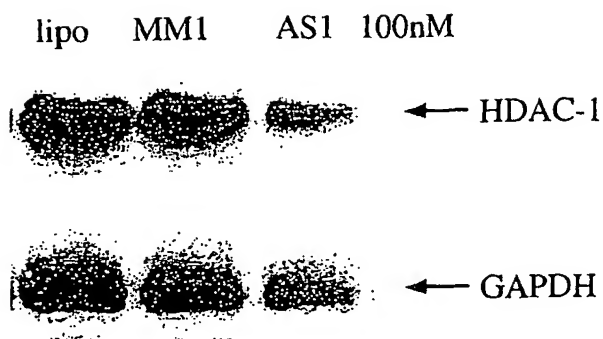


FIG. 9A

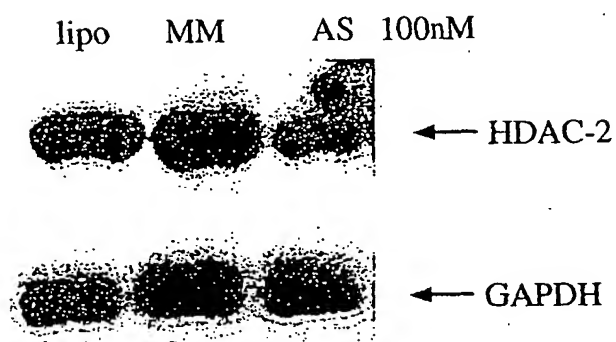


FIG. 9B

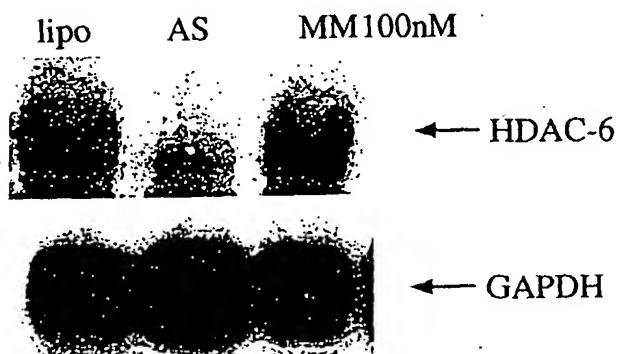


FIG. 9C

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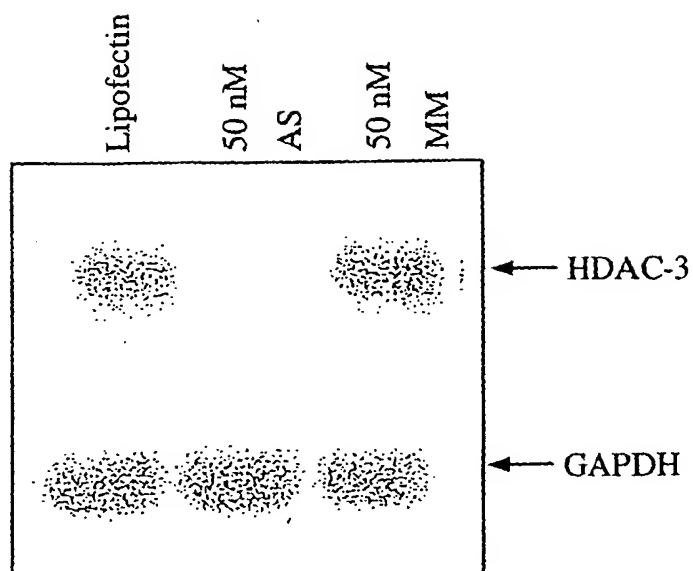


FIG. 9D

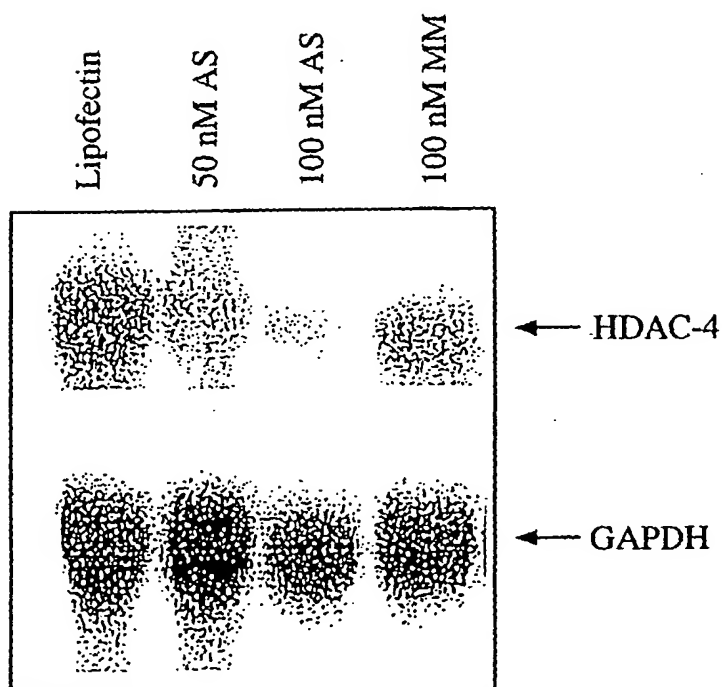


FIG. 9E

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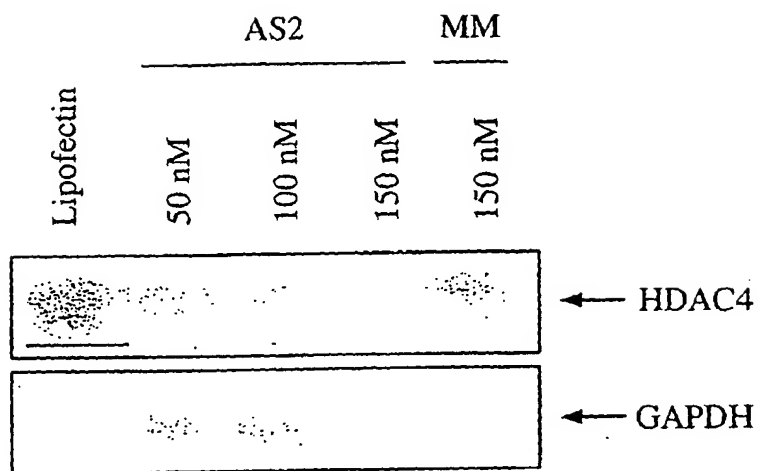


FIG. 9F

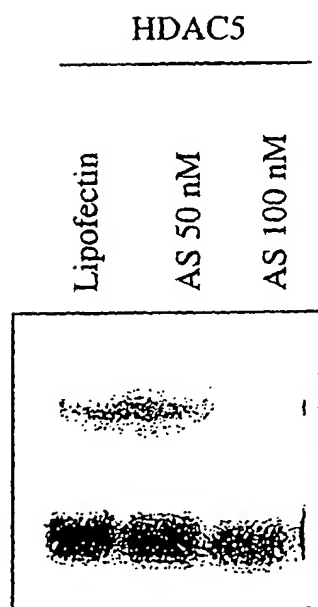


FIG. 9G

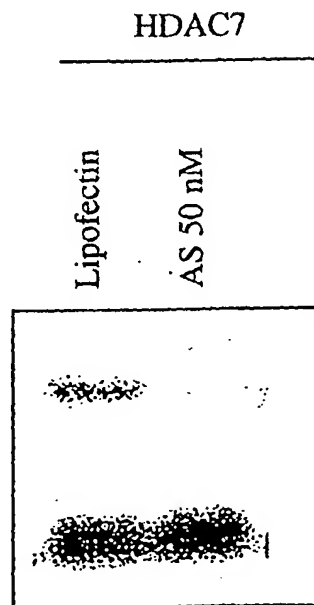


FIG. 9H

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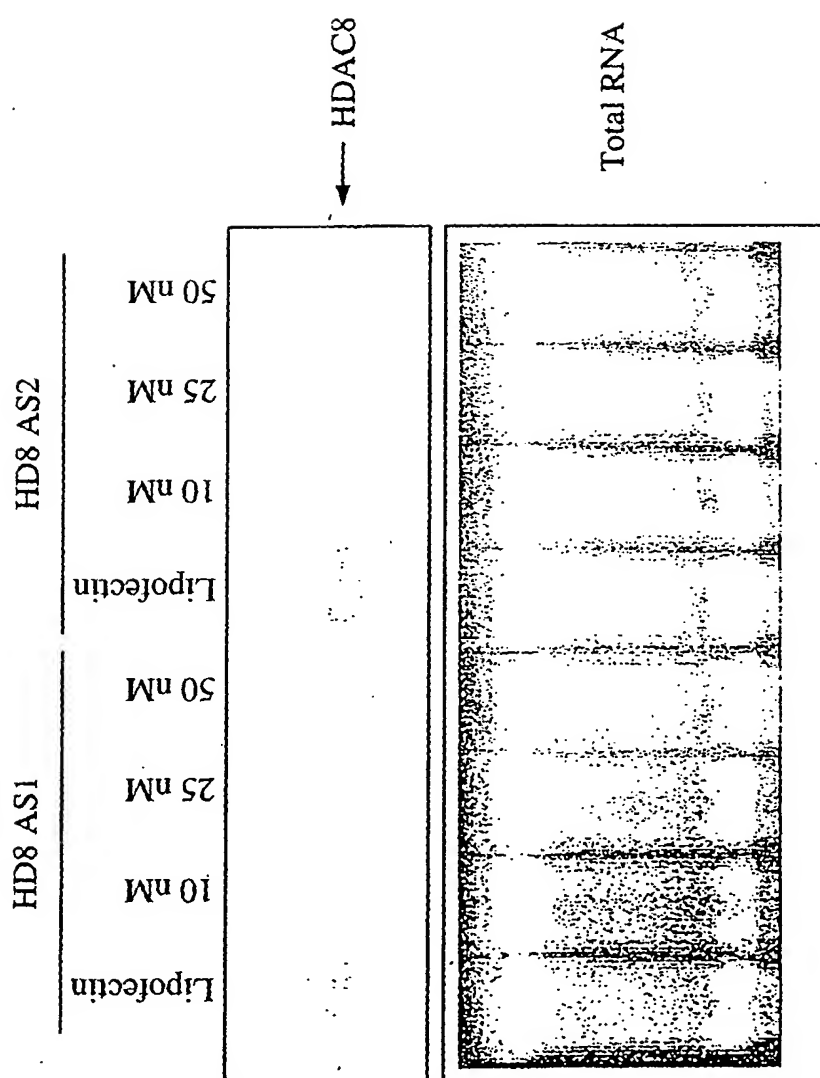
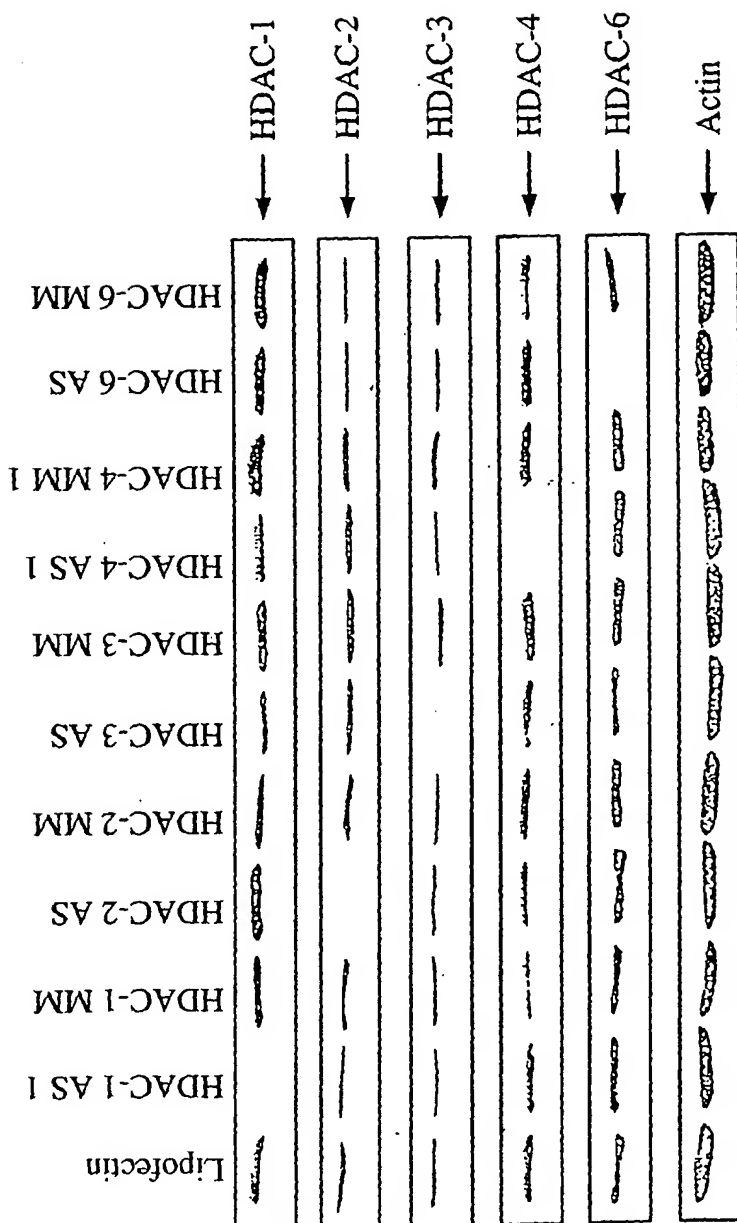


FIG. 9I

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AS = Antisense
 MM = Mismatch
 NS = Non-specific control
 3 day treatment
 Oligonucleotide conc - 50nM

FIG. 10A

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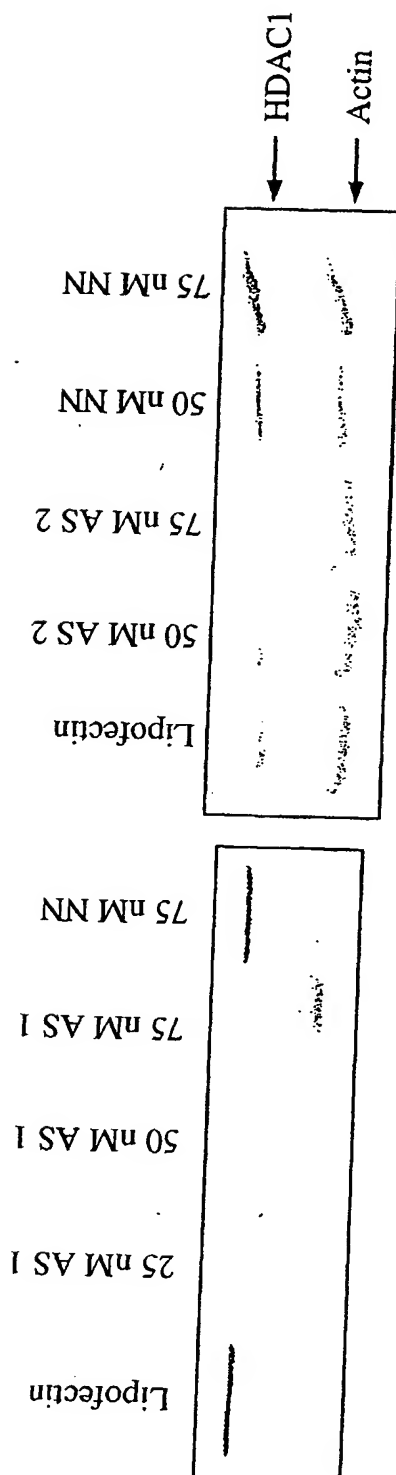


FIG. 10B

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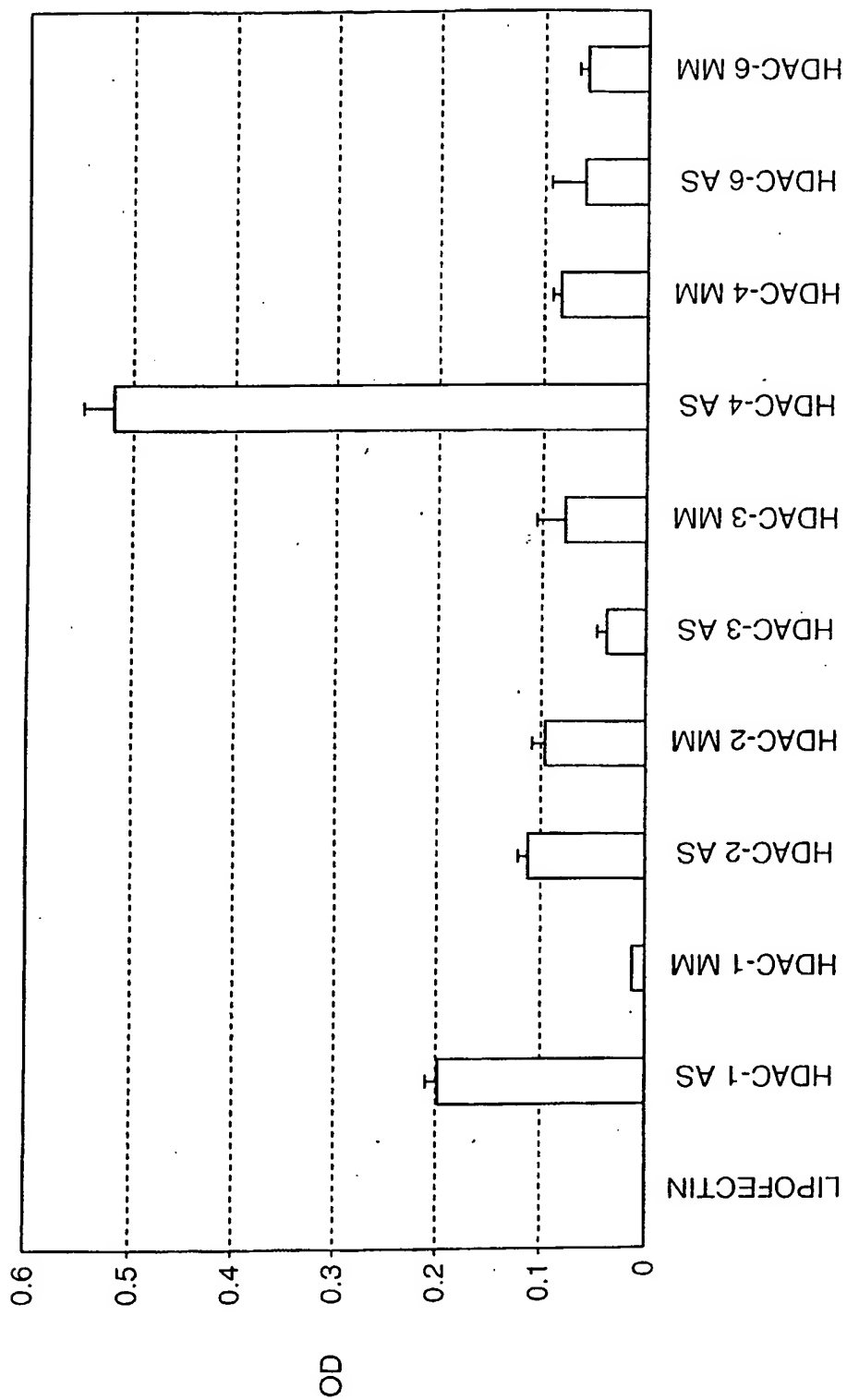


FIG. 11

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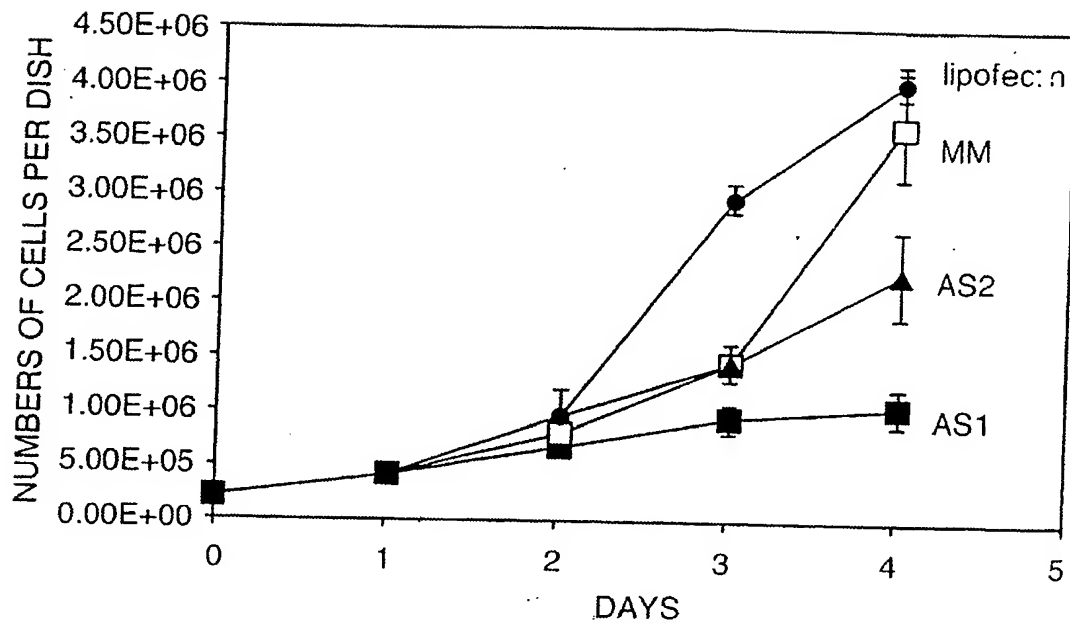


FIG. 12A

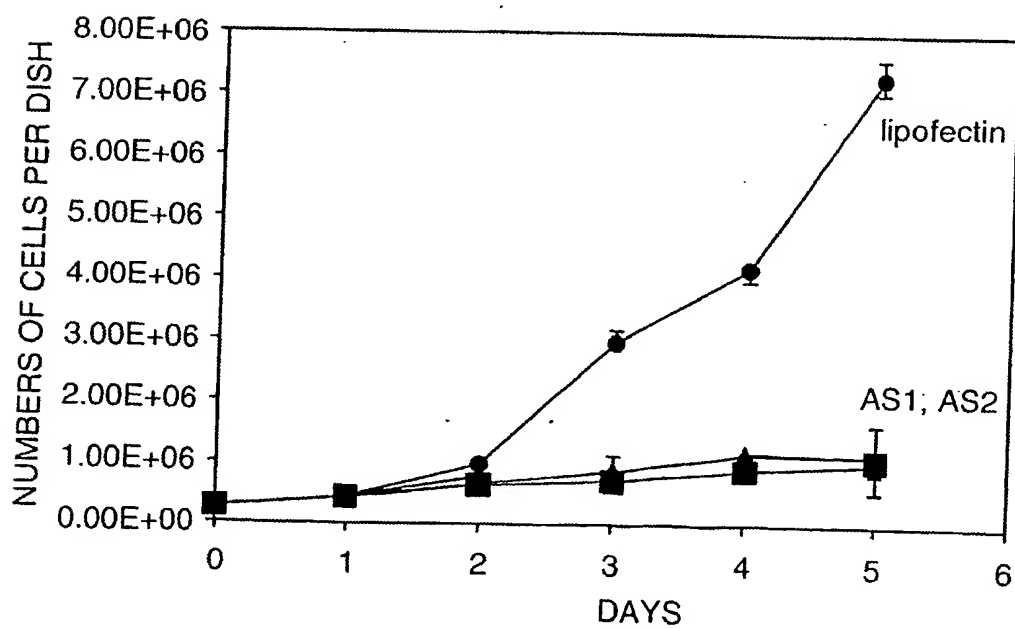


FIG. 12B

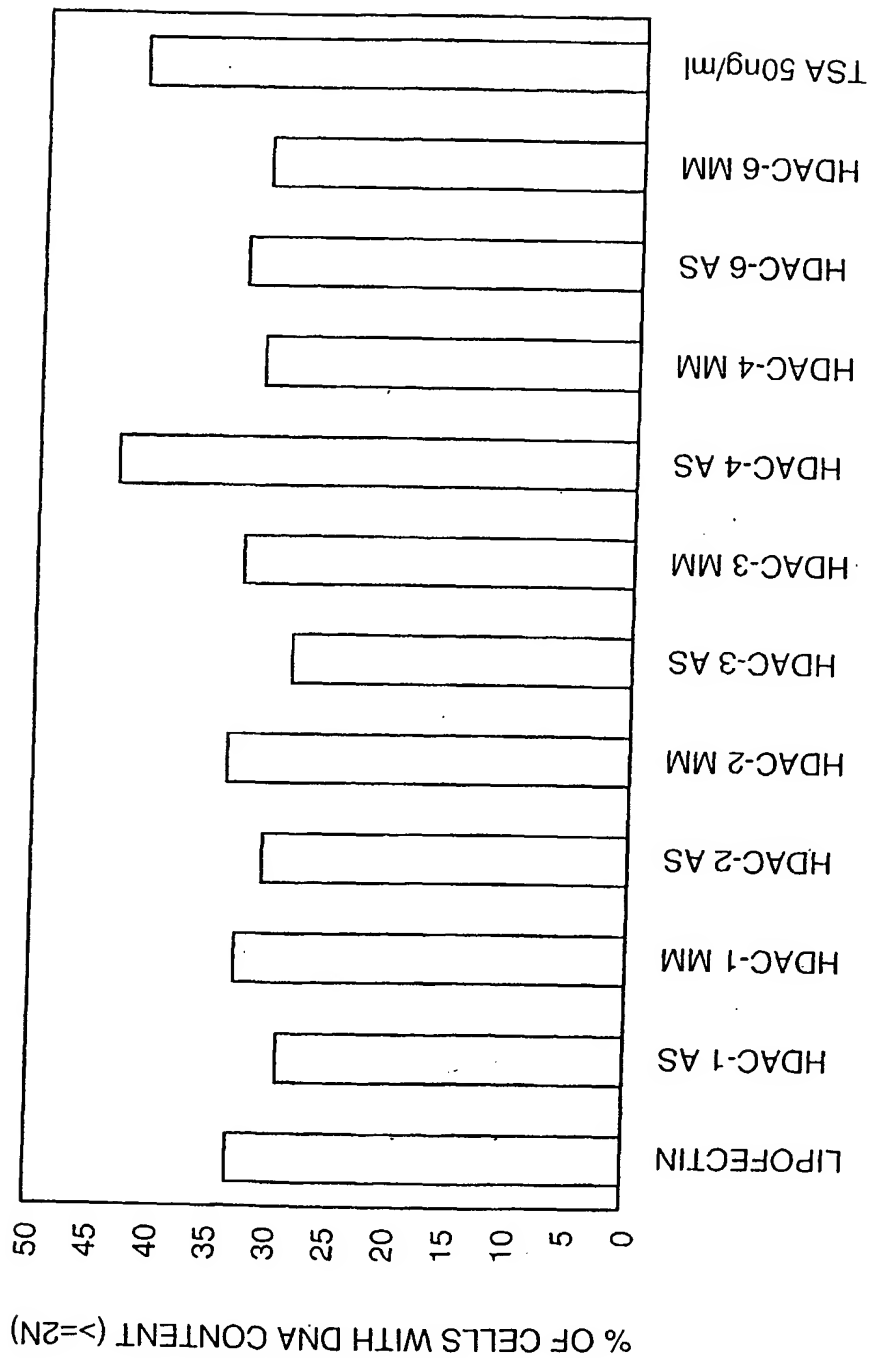


FIG. 13

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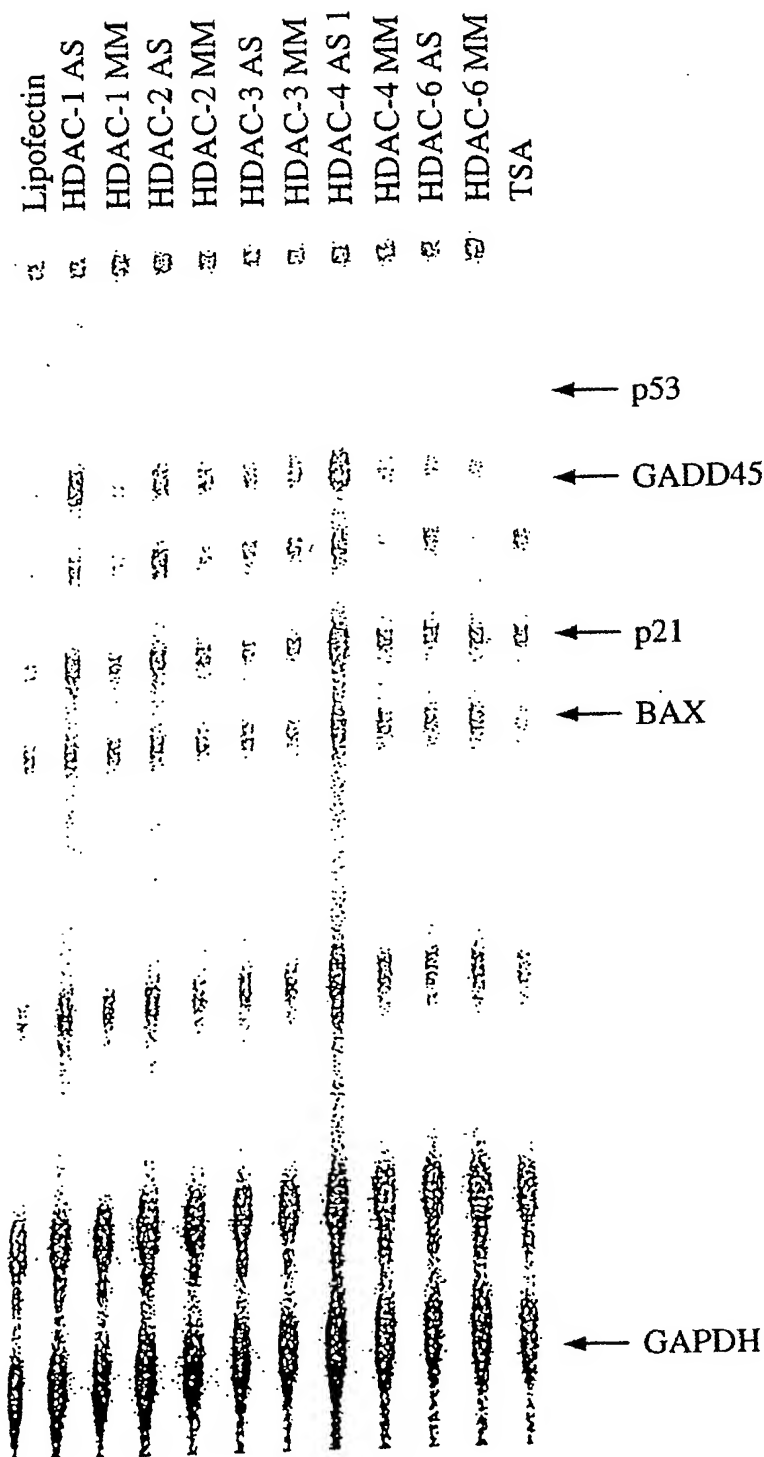


FIG. 14

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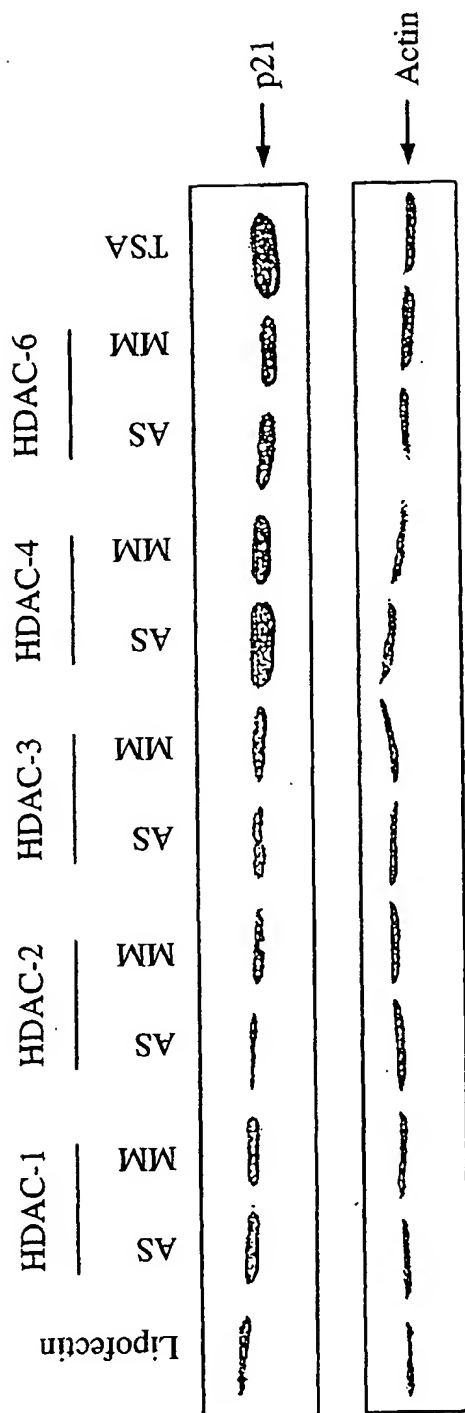


FIG. 15

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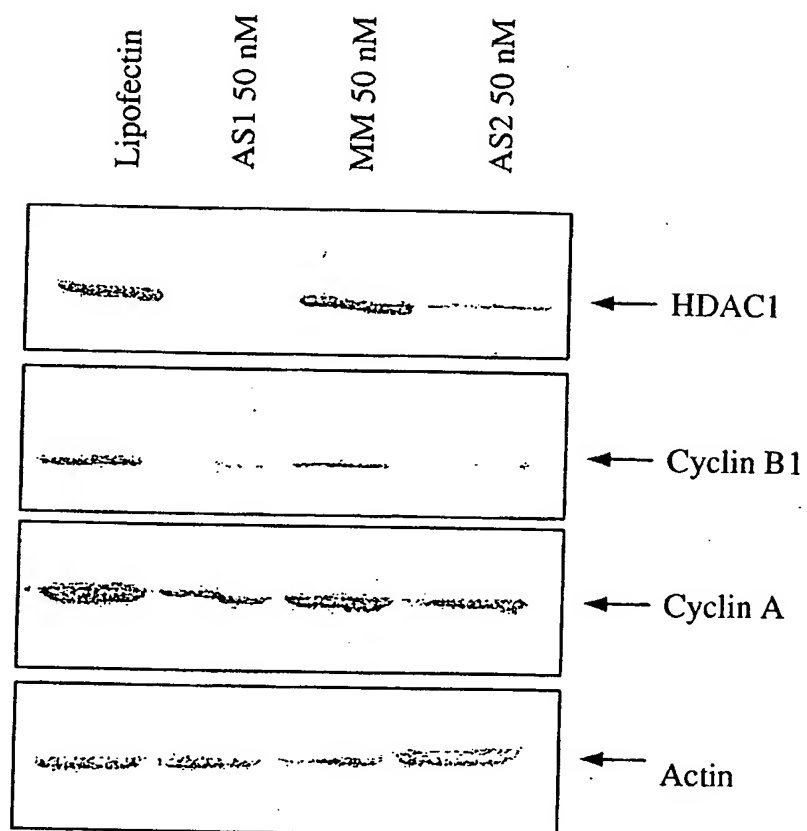
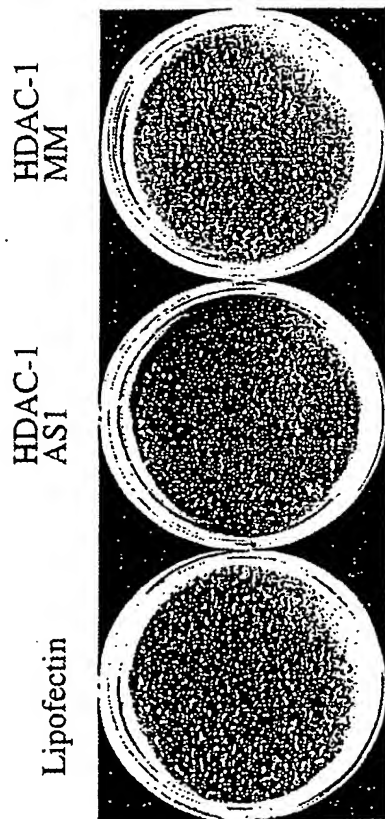


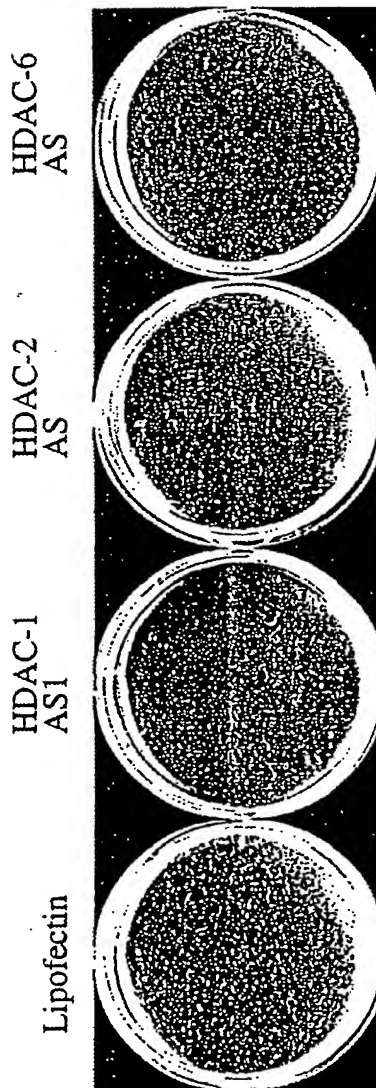
FIG. 16

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Colony Numbers -1200 -120 -1160

FIG. 17A



Colony Numbers -1200 -120 -890 -730

FIG. 17B

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau

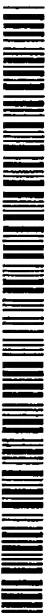


(43) International Publication Date
23 January 2003 (23.01.2003)

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(10) International Publication Number
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- (51) International Patent Classification⁷: C12N 15/11, A61K 31/7125, C07H 21/04, C12Q 1/44 // A61P 35/00
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- (30) Priority Data:
60/192,157 24 March 2000 (24.03.2000) US
- (71) Applicant: METHYLGENE, INC. [CA/CA]; 7220 Frederick-Banting, St. Laurent, Quebec H4S 2A1 (CA).
- (72) Inventors: LI, Zuomei; 22 Oriole Street, Kirkland, H9H 3x3 (CA). BONFILS, Claire; 10629 Rue St. Hubert, Montreal, Quebec H9X 3V3 (CA). BESTERMAN, Jeffrey; 51 Gray Crescent, Baie d'Urfe, H9X 3V3 (CA).
- (74) Agents: COTE, France et al.; Swabey Ogilvy Renault, 1981 McGill College Ave. - Suite 1600, Montréal, Québec H3A 2Y3 (CA).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
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- (88) Date of publication of the international search report:
13 May 2004
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 2003/006652 A3

(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/11 A61K31/7125 C07H21/04 C12Q1/44 //A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 97 35990 A (JAMISON TIMOTHY F ;HARVARD COLLEGE (US); TAUNTON JACK (US); HASSIG) 2 October 1997 (1997-10-02)</p> <p>page 5, line 8 -page 6, line 27</p> <p>page 27, line 13 -page 29, line 2</p> <p>page 48, line 15 -page 65</p> <p>claims; examples</p> <p style="text-align: center;">--- -/-</p>	<p>1-3,6-8, 26-48</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

28 February 2003

Date of mailing of the international search report

06/03/2003

Name and mailing address of the ISA

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Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOSHIDA M ET AL: "POTENT AND SPECIFIC INHIBITION OF MAMMALIAN HISTONE DEACETYLASE BOTH IN VIVO AND IN VITRO BY TRICHOSTATIN A" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 28, 5 October 1990 (1990-10-05), pages 17174-17179, XP000616087 ISSN: 0021-9258 cited in the application the whole document	1,26,45
A	--- ZHAO Q ET AL: "EFFECT OF DIFFERENT CHEMICALLY MODIFIED OLIGODEOXYNUCLEOTIDES ON IMMUNE STIMULATION" BIOCHEMICAL PHARMACOLOGY, vol. 51, no. 2, 26 January 1996 (1996-01-26), pages 173-182, XP000610208 ISSN: 0006-2952 the whole document	4,5,9
P,X	--- WO 00 71703 A (METHYLGENE INC) 30 November 2000 (2000-11-30) the whole document	1-11, 26-48
P,X	--- WO 00 23112 A (BESTERMAN JEFFREY M ;MACLEOD ALAN ROBERT (CA); METHYLGENE INC (CA)) 27 April 2000 (2000-04-27) examples 9,10 page 29; tables 2,3 claims 38-50	1-12, 26-37, 44-48
E	--- WO 01 70675 A (METHYLGENE INC) 27 September 2001 (2001-09-27) page 46 -page 54; table 1 page 68; example 13 page 203 -page 223; examples 159-162 claims	1-16, 24-37, 44-48

INTERNATIONAL SEARCH REPORT

international application No.
PCT/IB 01/02907

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 26-33 (as far as in vivo methods are concerned) and claims 34-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 17-23
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9,26-48 (all partially) and claims 10-11

An antisense oligonucleotide against HDAC1; modified forms thereof and its applications in therapy and diagnostic.

2. Claims: 1-9,26-47 (all partially) and claims 12-13

As for subject 1., but concerning HDAC2.

3. Claims: 1-9,26-47 (all partially) and claims 14-15

As for subject 1., but concerning HDAC3.

4. Claims: 1-9,26-48 (all partially) and claim 16

As for subject 1., but concerning HDAC4.

5. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC5.

6. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC6.

7. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC7.

8. Claims: 1-9,26-47 (all partially) and claims 24-25

As for subject 1., but concerning HDAC8.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17-23

The application as filed does not comprise claims 17 to 23. Consequently only claims 1-16 and 24-48 have been taken into account.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 01/02907

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9735990	A	02-10-1997	AU 2990597 A WO 9735990 A2	17-10-1997 02-10-1997
WO 0071703	A	30-11-2000	AU 6718200 A EP 1173562 A2 WO 0071703 A2 JP 2003500052 T	12-12-2000 23-01-2002 30-11-2000 07-01-2003
WO 0023112	A	27-04-2000	AU 6519499 A EP 1243289 A2 EP 1243290 A2 EP 1123111 A1 JP 2002528391 T WO 0023112 A1	08-05-2000 25-09-2002 25-09-2002 16-08-2001 03-09-2002 27-04-2000
WO 0170675	A	27-09-2001	AU 4870101 A EP 1280764 A2 WO 0170675 A2 US 2002115826 A1	03-10-2001 05-02-2003 27-09-2001 22-08-2002

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